Response

Applicant's Comments Regarding Amendments

Claims 1 and 22 are presently amended to clarify that the target gene of interest in Applicant's invention in the human cell is necessarily "mammalian," as opposed to an artificially introduced marker gene that is otherwise not found in the same genus as the cell. This is supported by Applicant's examples in the specification. Similarly, amendments are also presently made to claims 23 and 24 in response to Examiner's Rejection under 35 U.S.C. §112, 2nd paragraph. Claims 9, 11, 21, 26, and 27 are amended in response to the Examiner's claim objections, but represent only formatting issues. No new matter has been added.

In the following discussions, Applicant presents arguments and evidence in support of his position. Some of the evidence is based on the Declaration of Dr. Alan Gewirtz, ("Gewirtz § 1.132 Declaration") entered into the prosecution record under 37 C.F.R. § 1.132 on September 19, 2005. A second Declaration, attached hereto is newly presented under 37 C.F.R. § 1.131 ("Declaration of Dr. Alan Gewirtz for Earlier Invention Under § 1.131 Declaration"), to be entered into the prosecution record.

1. Claim Rejection - 35 U.S.C. § 112, first paragraph, written description.

The Examiner has rejected all pending claims (claims 1, 2, 5, 7-9, 11, 21-27) under 35 U.S.C. § 112, first paragraph as failing to comply with the written description requirement, maintaining the rejection of October 6, 2004 and April 23, 2005, despite Applicant's repeated arguments and amendments, including in-person discussions on July 18, 2005. Applicant respectfully traverses the Examiner's conclusions and asserts that the Examiner has failed to demonstrate that one of ordinary skill in the art would question that Applicant was in possession of his invention as demonstrated by the written description of the invention at the filing date of the patent application.

The Examiner has withdrawn the rejection that the specification provides insufficient enablement of claims 1, 2, 5, 7-9, 11, 21 and 22 *in vitro* methods under 35 U.S.C. § 112, first paragraph, yet the Examiner continues to tie the present rejection to the enablement requirement, stating at page 5 of the Action that "the potential enablement of a particular and narrow method within the broad genus of methods now claimed, does not provide adequate written description, commensurate in breadth, with the claimed genus." In other words, while offering no evidence to support this rejection, and providing only unsubstantiated conclusions based upon his own

understanding of the art, the Examiner appears to have determined that regardless of Applicant's teachings in light of the state of the art at the time of the invention, only narrow claims to the expressly exemplified disrupted human target gene were adequately described.

The test for the adequacy of the written description of a patent application is whether the disclosure reasonably conveys to a person skilled in the art that the inventor had possession of the claimed subject matter at the time of the original filing date. Contrary to the Examiner's position, however, the law does not require Applicant to *literally* describe the claimed subject matter in exactly the same terms as those that are used in the claims. It must simply indicate to persons skilled in the art that as of the effective filing date, Applicant had invented what is now claimed.

There can be little doubt that Applicant has, indeed, demonstrated as described in the specification, that the expression of a target gene has been disrupted in *vitro* at the mRNA level in a mammalian gene in a human cell by the claimed methods. Thus, it is surprising that the Examiner has maintained this rejection, particularly given that the courts have generally taken the position that, absent evidence to the contrary, an adequate written description of the claimed invention is present in the specification as filed. Yet, the Examiner makes a circular argument – first apparently agreeing that there is a strong presumption by the courts that there is an adequate written description of the claimed invention in the specification as filed – but then stating that an adequate written description of the claimed invention is *only* a presumption. A presumption has the effect of shifting the burden of going forward with evidence, not the burden of persuasion. *A.C. Aukerman Co. v. R.L. Chaides Construction Co.*, 22 USPQ2d 1321, 1324-5 (Fed. Cir. 1992)(*en banc*). But, the Examiner has offered no evidence to support his conclusion that "the skilled artisan would not recognize that Applicant was in possession of the method, commensurate with the breadth of what is now claimed." As stated, for example, in *Ex parte Sudilovsky*, 21 USPQ2d 1702, 1703 (BPAI 1991), "it is incumbent upon the Patent Office, whenever a rejection on this basis is made, to explain *why* it doubts the truth or accuracy of any statement in a supporting disclosure and to back up assertions of its own with acceptable evidence on reasoning which is inconsistent with the contested statement." Yet no such evidence has been presented by the Examiner.

The Examiner further states that the rejection is not based upon whether "the skilled artisan would recognize what is meant by an RNA that is homologous to the target gene" and

apparently accepts that the meaning of the phrase is not in question. Rather, the Examiner asserts that maintaining the rejection is proper because "the disclosure of the specification and the teachings of the prior art as a whole, fail to provide or point to the structure of an RNA that can have a degree of homology to any target gene." However, this is a mischaracterization of Applicant's claim. Nowhere does Applicant claim that a selected RNA is homologous to any target gene, and to make such a statement is not representative of what one of ordinary skill in the art at the filing date of Applicant's application would have understood the written description in support of the claims to mean.

The record shows that the Examiner has stated that the specification enables the use of RNAi *in vitro* to practice methods of disrupting expression of a human KitR gene in human cell lines using Kit dsRNA (KdsRNA). See, Examiner's statement in Office Action mailed May 23, 2005, at page 6, paragraph 6. Accordingly, Applicant's specification teaches the degree of homology for the dsRNA used in the claimed invention, as well as the range of sizes for the dsRNA (from 17 to about 830 bp) and the method of making dsRNA homologous to the target gene (see, *e.g.*, specification at page 1; line 30 through page 2, line 28; and page 12, line 28 through page 13, line 12). In other words, Applicant could not have tested the methods of his invention and described them in the specification if he had not been in possession of the invention. Specifically, Applicant describes that in the exemplified embodiment, two strands of RNA were made in an *in vitro* transcription reaction using a DNA template comprising a fragment of the target gene subcloned into an expression vector having an SP6 promoter and a T7 promoter. The *in vitro* transcription reactions using the cognate RNA polymerases were followed by strand annealing and purification, thus yielding a dsRNA molecule homologous to the target gene. Moreover, one could readily calculate the degree of homology of a selected dsRNA to its target gene. See Applicant's record.

In the examples, a representative long dsRNA molecule of 828 bp (see page 12, lines 28-31) homologous to cKitR was employed in the method to induce RNAi. This number, therefore, provides an upper limit to the size of the dsRNA that was successfully used in Applicant's method. A lower size limitation can be deduced from the size of the human genome. One skilled in the art would know that any sequence that is 17 nucleotides or longer has a high probability of being unique. Since the mechanism of RNAi is that each dsRNA is homologous to a particular target sequence (as opposed to the one size fits all target genes interpretation of the Examiner), one skilled in the art knows that the minimum dsRNA size to achieve target specificity is 17 nucleotides.

Therefore, the dsRNA that would be effective in Applicant's invention ranges from 17 to about 830 base pairs. For support of these calculations, see Applicant's prosecution record.

While the written description must communicate that which is needed to enable the skilled artisan to make and use the claimed invention, it is well established that a patent applicant is entitled to claim his invention generically, when he describes it sufficiently to meet the requirements of §112. *See, e.g., Utter v. Hiraga* 6 USPQ2d 1709, 1714 (Fed. Cir. 1988) (A specification may, within the meaning of 35 U.S.C. 112, first paragraph, contain a written description of a broadly claimed invention without describing all species that the claim encompasses), and *U.S. v. Telectronics, Inc.*, 8 USPQ2d 1217, 1223 (Fed. Cir. 1988) (The law does not require an applicant to describe in his specification every conceivable embodiment of the invention).

Therefore, Applicant has provided adequate written description to meet the requirements of §112. There is a clear description provided in Applicant's specification of both the preparation of the dsRNA molecule that was homologous to the exemplified target human gene and its size, as well as the calculation of degree of homology to the target gene, as used in Applicant's methods. As a result, based upon the disclosure in the specification as filed, and the knowledge imputed to the skilled artisan at the time of the invention, a skilled artisan would indeed understand that Applicant had possession of the claimed subject matter. Therefore, Applicant respectfully requests that the rejection of all pending claims under 35 U.S.C. §112, first paragraph, regarding Applicant's written description be reconsidered and reversed.

2. Claim Rejections - 35 U.S.C. § 112, second paragraph

The Examiner has rejected claims 23-27 under 35 U.S.C. § 112, second paragraph, as being indefinite for failing to particularly point out and distinctly claim the subject matter which Applicant regards as the invention. Applicant respectfully traverses this rejection, and asserts that the Examiner has not applied the objective "person having ordinary skill in the art" test to the claims, and has ignored the clear disclosure of the specification.

Rejections of this type are appropriate only where Applicant has indicated, somewhere other than in the Application as filed, that the invention is something different from what is defined by the claims. In other words, the invention set forth in the claims must be presumed, in the absence of evidence to the contrary, to be that which applicants regard as their invention. *In re Moore,* 439 F.2d 1232, 169 USPQ 236 (CCPA 1971). Yet, the Examiner provides no

evidence that Applicant has anywhere stated or indicated that the invention is something different from what is defined by the claims.

Further, MPEP 2173.02 states that "Examiners are encouraged to suggest claim language to the applicants to improve the clarity or precision of the language used, but should not reject claims or insist on their own preferences if other modes of expression selected by the applicant satisfy the statutory requirement." In that regard, if the Examiner offers suggested language, Applicant will certainly consider making changes that will improve clarity, but can at this point see no such changes, and the Examiner has offered none.

When determining whether or not a claim meets the statutory requirement for definiteness the claim must not be analyzed in a vacuum. The test for definiteness under 35 U.S.C. §112, second paragraph is whether, "those skilled in the art would understand what is claimed when the claim is read in light of the specification." *Orthokinetics, Inc. v. Safety Travel Chairs, Inc.*, 806 F.2d 1565, 1 USPQ2d 1081 (Fed. Cir. 1986).

Of course, in this situation, the rejected claims 23-27 are all directly or indirectly dependent on claim 22, although claim 22 was not itself rejected under 35 U.S.C. §112, second paragraph, as noted by the Examiner. In making this rejection, the Examiner states that "claim 23 is drawn to the method of claim 22, further comprising initiating RNAi in a population of the human cells using KdsRNA. However, the Examiner continues that "the scope of the relationship of claim 23 to claim 22 cannot be determined without assumption because the term "further comprises" indicates that the method of claim 23 is the entire method of claim 22 plus an additional provision of the KdsRNA to initiate RNA interference."

In response, Applicant respectfully submits that the Examiner has misconstrued claim 23 in its relationship to claim 22. For easier comparison, the two claims are reproduced at this point, including the amended changes to claims 22 and 23.

> 22. A method for disrupting expression of a mammalian target gene *in vitro* at the mRNA level in a human cell, wherein the method comprises providing small interfering RNA guide sequences which are homologous to a portion of the target gene, such that RNAi of the target gene is induced.
>
> 23. The method of claim 22, wherein the method further comprises providing to the human cell an effective amount of KdsRNA as the interfering RNA guide sequence to initiate RNA interference, thereby effecting disruption of gene expression of KitR when it is the target gene in the cell.

Claims are, of course, carefully drafted not only to define the invention, but also to protect against infringement. In order to infringe an independent claim, an accused device must contain every element of the independent claim. In order to infringe a dependent claim (in this case, claim 23), a device must contain every element of the dependent claim plus every element of every claim from which it depends (in this case, claim 22). When properly drafted, the dependent claim should show a meaningful narrowing of the invention that is broadly defined in the independent claim from which it depends. The Examiner does not appear to be a questioning Applicant's claim 22. As asserted by the Examiner, claim 23, is a proper dependent claim including all of claim 22, plus the provisions of claim 23, and it does exactly that. If claim 23 were rewritten to in independent form it would read: "A method for disrupting expression of a mammalian target gene *in vitro* at the mRNA level in a human cell, wherein the method comprises providing small interfering RNA guide sequences which are homologous to a portion of the target gene, such that RNAi of the target gene is induced, and wherein an effective amount of KdsRNA is introduced into the cell to initiate the RNA interference, thereby effecting disruption of expression of the target gene in the cell, KitR."

Thus, the dependency of one claim (specific for disrupting the expression of the KitR gene as a target in the human cell), as it relates to the independent claim (defining the method of initiating RNAi inhibition of expression of a mammalian target gene to which the introduced dsRNA is homologous in the human cell, in general, without naming the target gene) seems proper to Applicant. However, there could be some confusion because claim 22 refers to the activity in a cell, whereas claim 23 refers to a population of human cells, and Applicant will endeavor to correct that discrepancy.

However, contrary to the Examiner's interpretation, Applicant can find no discussion in either claim of "multiple administrations of different double stranded RNAs." Nothing is even suggested about repeated or multiple administrations in any claim, and Applicant is at a loss as to what the Examiner may be referring to. The term "further comprising" is proper in claim 23, meaning all of the provisions of claim 22, reiterated in claim 23, plus the further limitations that are provided regarding a specifically identified Kit target gene. If, however, the Examiner can suggest a better way to write these claims, Applicant would be willing to discuss. It must be noted, however, that for the sake of moving this patent to allowance and use by the public, at some point these continued requests for further amendments must stop, even if the clarity of the

claims is not perfect in the Examiner's eyes. It need only be clear to one of ordinary skill in the art, and Applicant has met that burden.

In the following sections, Applicant presents additional arguments and evidence in support of the position that claims 23-27 do, in fact, particularly point out and distinctly claim the subject matter which Applicant regards as his invention. Applicant respectfully submits that one of ordinary skill in the art would, indeed, be informed of what is claimed when the claims are read in light of claim 22, and the specification.

Accordingly, Applicant respectfully requests that the Examiner's rejection of claims 23-27 under 35 U.S.C. §112, second paragraph be reversed.

A. Applicant's Response to the Examiner's "Response to Arguments"

Applicant respectfully submits that the Examiner's statements under the heading "Response to Arguments" on p. 4 of the Office Action mailed on February 6, 2004, concerning the adequacy of Applicant's written description are in error. The Applicant offers the previous subsections in this Section 2 in support of Applicant's position. The Examiner concurs that the courts have supported a strong presumption that the specification provides an adequate written description of what is claimed – but states that it is *only* a presumption, and facts are required to support that it can be reasonably determined that Applicant was in possession of the claimed invention. To the contrary, however, it is respectfully submitted that the burden is not on the Applicant to support the presumption. Overcoming a strong presumption falls to the party arguing against the presumption, and must be supported by facts, not merely unsupported conclusions. See, *Ex parte Sudilovsky*, 21 USPQ2d 1702, 1703 (BPAI 1991), as discussed above.

Applicant has provided a full and complete description of his claimed invention in the specification, including actual examples to demonstrate its operation as claimed, thereby establishing the presumption of an adequate written description. Thus, the burden to overcome such a presumption is on the Examiner to provide a factual basis for disregarding the specification and asserting that one of ordinary skill in the art with knowledge in the field of molecular/cellular biology would not believe that Dr. Gewirtz had possession of the invention commensurate with the pending claims. This, the Examiner has failed to do.

In making his Response to Applicant's Arguments, the Examiner agrees that one of ordinary skill in the art would understand what is meant by RNA that is homologous to the target gene." However, the Examiner asserts that Applicant's specification in light of the prior art

would not teach or "point to the structure of an RNA that can have a degree of homology to *any* target gene . . . by RNA interference, *in vitro* in *any* human cell." The Examiner continues by acknowledging that Applicant has provided, not only an example in the specification that demonstrates the degree of homology needed for RNAi in a human cell, but a discussion of error rates and upper and lower length limitations of a dsRNA homologous to a target gene. In addition, the Examiner agrees that Applicant has disclosed a variety of methods for obtaining the dsRNA. Nevertheless, the Examiner makes the baseless assertion that Applicant failed to provide argument with regard to the teaching of the various known ways of obtaining the dsRNA. In response to this statement Applicant reiterates that it is not his burden to further prove or argue what the Examiner seems to agree are adequate ways of obtaining dsRNA.

Applicant has met his requirement and taught methods for the preparation and use of the claimed invention (acknowledged by the Examiner in his withdrawal of the lack of enablement rejection for the subject claims). How could the invention, in fact, be enabled by Applicant and by Applicant's specification unless an adequate written description had been provided for how to make and use the invention? In addition, the Examiner has found prior art that he asserts renders Applicant's claimed invention obvious, which would not be possible if the invention were truly indefinite. Since the adequacy of the written description is measured in light of the prior art, it seems incomprehensible that the Examiner could conclude that Applicant's claimed invention lacks written description – yet at the same time the Examiner has found that art prior to Applicant's invention would provide an adequate teaching of how to make and use the claimed invention.

The Examiner's contentions are unsupported by facts, and lack a basis for questioning or overturning the presumption that Applicant's written description is, indeed, adequate under 35 U.S.C. §112, 1st paragraph, written description requirement. As a result, Applicant asserts that the Examiner's reasoning regarding his burden of proof, as well as, the extent of what would be understood by one of ordinary skill in the art based upon the specification as filed, is erroneous, and should properly be withdrawn. Absolutely no facts have been provided in the Examiner's arguments as to why Applicant's claims should be limited to *only* the example(s) provided in the specification, and nowhere do US patent laws require such a limitation. To the contrary, as pointed out above, Applicant is entitled to the breadth of his invention, and examples are just that – exemplary of a best mode of practicing the invention. The Example is not, and should not be,

limiting – absent a factual showing to the contrary - facts which are missing from the Examiner's Response. Accordingly, Applicant respectfully requests that all §112 rejections be reconsidered and reversed.

3. Claim Rejections - 35 U.S.C. §102(a) re: Kreutzer (WO 00/44895).

Anticipation under §102(a) requires that the identical invention that is claimed was previously known to others, and thus is not new. *Continental Can Co. USA Inc. v. Monsanto*, 20 USPQ2d 1746, 1748 (Fed. Cir. 1991). However, neither of the references cited by the Examiner is anticipatory of Applicant's claimed method for disrupting expression of a mammalian target gene at the mRNA level in a human cell, wherein the method comprises initiating RNA interference (RNAi) *in vitro*

The Examiner has rejected claims 1, 2, 5, 7-9, 11, and 21-22 under 35 U.S.C. § 102(a) as being anticipated by Kreutzer (WO 00/44895). In making this rejection, the Examiner asserts that Kreutzer discloses "inhibiting target gene expression in a mammalian cell *in vitro* . . . using a short dsRNA (no more than 49 bp in length) homologous to a portion of the target gene in a pharmaceutical composition," wherein the *in vitro* cells "can be human cells." However, there are several flaws in this logic.

First and foremost under 35 U.S.C. §102(a), the Examiner is asserting that Applicant's claimed invention was known and used by another in this or a foreign country prior to Applicant's date of invention. The Kreutzer PCT publication is submitted in German, without English language translation. Nevertheless, the prior art document submitted as a "printed publication" under 35 U.S.C. §102(a) is effective against Applicant only as of the publication date of August 3, 2000, but that date does not prove the truth of the matters addressed in the document, nor does it prove that the cited document teaches or anticipates Applicant's invention. See, *e.g., Continental Can Co. USA Inc. v. Monsanto*, 20 USPQ2d 1746, 1748 (Fed. Cir. 1991).

Applicant's effective date of invention is November 14, 2000, the filing date of U.S. Provisional Application No. 60/248,346, but he conceived of the invention and diligently worked to reduce it to practice prior to August 2000, as demonstrated by copies of laboratory notebook pages attached hereto in support of a Declaration of Dr. Alan Gewirtz for Earlier Invention Under 35 U.S.C. §1.131. Accordingly, because the difference between the two dates is less than one year, Applicant is entitled to swear behind the date of the cited publication by a Declaration demonstrating earlier invention. See, Declaration of Dr. Alan Gewirtz for Earlier Invention

Under §1.131, attached hereto at Tab 2. Consequently, having demonstrated that Applicant's date of invention *preceded* the publication of the cited Kreutzer (WO 00/44895) reference, the rejection under 35 U.S.C. §102(a) is moot, and Applicant asks that it be reversed.

Secondly, in addition to Applicant's §1.131 Declaration, the Kreutzer reference fails to provide evidence of each and every element of Applicant's claimed invention and, therefore, it fails to provide anticipation. In accordance with Applicant's claimed invention, dsRNA is used to initiate disruption of the expression of a mammalian gene in a human cell. Kruetzer on the other hand, teaches a method that is quite different from Applicant's, even though both involve dsRNA. Kruetzer demonstrates the effectiveness of his invention by microinjecting the dsRNA into cells of a mouse fibroblast line. This marks the first point of distinction between Kruetzer practiced in a mouse cell and Applicant's invention taught in a human cell. This is a highly unpredictable art, and one of ordinary skill in the art knows that results in mice or in mouse cells do not always perform in the same manner in a human or human cell.

Mouse models of human disease pathogenesis have become a central part of many types of biomedical research. But over time, many have questioned how faithful is the mimicry of human diseases in mice. Mice are not simple small people. Humans are about 3,000 times larger than mice and are formed from a proportionately larger number of cells. Moreover, humans live, on average, 30–50 times longer than mice, meaning that humans undergo about 10^5 more cell divisions in a lifetime. As a result, human cells have adapted certain intrinsic mechanisms that are not found in mice – and created differences between the cellular responses in the two species. Discrepancies in the organization of cell-autonomous regulatory pathways indicate markedly different courses of tumor progression in the two species. In addition, the substantially greater number of pathways that are required to transform human cells means that most of the cell-autonomous, anticancer protective mechanisms that are present in human cells must have been developed, or at least perfected, during the time since our evolutionary lineage diverged from that of rodents. Consequently, conventional mouse knockout models of *human* tumor-suppressor genes often display a tumor spectrum *at variance with the human pathology.* For instance, *mouse models have often failed* to accurately reproduce the initiation of many sporadic *human* tumors, which seems to involve the stochastic activation of an oncogene in a single cell that is surrounded by an otherwise normal tissue microenvironment – cells of the type that would be likely targets of RNAi. *See, e.g.,* Rangarajan and Weinberg, "Comparative

Biology of Mouse Versus Human Cells: Modeling Human Cancer in Mice," in *Perspectives, Nature Reviews, Cancer* 3:952 (Dec. 2003), also available at www.nature.com/reviews/cancer (Reference is not prior art and is not cited in an IDS). As another example, an inverse correlation has been found between the lifespan of a species and the rate of DMBA (dimethyl benz(a)anthracene) carcinogen binding to DNA, reflecting the differential rate of activation of the DMBA pro-carcinogen into its actively mutagenic diol epoxide form. *Id.* So, cultured *rodent* fibroblasts, such as those used by Kreutzer, bind activated DMBA to their DNA very efficiently, whereas cultured *human* fibroblasts do so much less efficiently. *Id.*

As a second point of distinction, while Kreutzer disrupted target gene expression at the mRNA level in a mouse fibroblast cell using dsRNA, Kreutzer's target was not a mammalian gene nor endogenous to the mouse cell. The Kreutzer group demonstrates *only* the silencing of an "artificial" or exogenous gene (luciferase) that was transfected into the target mouse cells. One of ordinary skill in the art would recognize that the use of an artificial marker gene is often done to make it "easier" to achieve and visualize gene silencing. It is much more difficult to obtain dsRNA silencing of a targeted *mammalian* gene. This is well known to individuals skilled in the triple helix and nucleic acid repair arts, in which concepts are typically tested on "artificial" genes before attempting to modify the more difficult endogenous mammalian genes.

Accordingly, Kreutzer comes up short of Applicant's invention, having tested the disclosed method *only* on the YFP luciferase gene that was artificially microinjected into the mouse cells. Kruetzer fails to disclose any RNA interference of a mammalian vertebrate gene, and certainly fails to provide any information that would provide one of ordinary skill in the art with a reasonable expectation of achieving success using RNAi to disrupt expression of a targeted mammalian gene in a human cell, regardless of whether Kruetzer hypothesizes that the *in vitro* cells "can be human cells, or not."

As a third point of distinction, the Kreutzer dsRNA is composed of two regions. Region I is complementary to the target mRNA and contains "not more than 49 nucleotides (bp)." Region I may be located anywhere within Region II, which is self-complementary. But Kreutzer does not describe Region II as complementary to the target mRNA! Region II consists of from 10 to 1000 nucleotides, which are autocomplementary, meaning that double stranded hairpin loops are formed which, contrary to Applicant's invention, lack the required "transcription of each is independently controlled to generate paired RNAs."

There is no apparent reason why the Kreutzer method works at all, and perhaps it does not work on a mammalian gene, but it certainly does not work in the same manner as Applicant's invention. The dsRNAs are structurally different - Applicant's invention does not have two regions of dsRNA - although the dsRNA that is complementary to the target gene is generally prepared in a similar manner (two complementary linearized strands of RNA to generate paired RNAs of defined length). The complementary region of Applicant's dsRNA to the mammalian target gene is not limited to ≤ 49 nucleotides, as is required by Kreutzer, making the dsRNA used in the two inventions structurally different. Moreover, the dsRNA is used differently as between Applicant's invention and Kreutzer, and therefore Kreutzer cannot anticipate Applicant's invention.

Applicant respectfully asserts therefore, that the Examiner's rejection of claims 1, 2, 5, 7-9, 11, and 21-22 under 35 U.S.C. § 102(a) is improper and asks that it be reversed and withdrawn.

4. Claim Rejections - 35 U.S.C. §102(e) re: Fire (US Patent No. 6,506,559).

The Examiner has maintained the rejected of claims 1, 2, 5, 7-9, 11, and 21-22 under 35 U.S.C. §102(e) as being anticipated by Fire (US Patent No. 6,506,559) for the reasons of record as stated in the Office Action mailed 5/23/05. Applicant has responded to the Examiner's assertions with regard to this rejection in excruciating detail as is evident in the record, yet in the Examiner's Response to Arguments, he states that while the arguments were considered, they were "not found to be persuasive." Applicant traverses this rejection and asserts that while the Examiner has properly presumed the Fire '599 patent to be valid under 35 U.S.C. §282, he has improperly has erroneously credited the issued Fire patent with far greater breadth than could possibly have been enabled by the limited teachings that were provided by the disruption of targeted gene expression that was demonstrated only in embryonic invertebrate cells.

The Fire patent (claimed U.S. effective filing date December 23, 1997, actual US filing date was December 18, 1998), discloses "a method for inhibiting expression of a target gene using double stranded RNA (dsRNA) to induce RNAi in a cell *in vitro* (col. 26, claim 1), wherein the cell is from *an* animal (col. 26, claim 6)." However, Fire teaches RNAi operations only in an embryonic nematode invertebrate cell from *C. elegans*. In making his argument, the Examiner states that "Fire *et al.* disclose that the cell with the target gene may be derived from or

contained in *any* organism (col. 8, line 13-14) and that examples of vertebrate animals include mammals and human (col. 8, lines 35-37)."

In fact, at the time of the '559 invention, Fire *et al.* knew *only* that the claimed method of using dsRNA inhibited expression only of a target gene in cells of an invertebrate embryo. As a result, Fire's specification contains data showing *only* the effect of dsRNA in embryonic invertebrate cells. Evidence shows that Fire had no idea whether the system would or could operate in any cell, other than an embryonic *C. elegans* cell and, in fact, there was strong evidence at the time to show why it would not operate in vertebrate cells based on the previously discussed defenses evoked in such cells. As has been previously pointed out in Applicant's record, in his own publications after his invention, Fire acknowledged that he did not actually believe that his claimed method would work in a human cell. See, *e.g.*, Montgomery and Fire, *TIG* 14:255-258 (1998), see, *e.g.*, page 258, sentence bridging columns 1 and 2 and page 258, first full paragraph, second column; Fire, *Trends Genet.* 15:358-363 (1999), (each is previously cited in recorded arguments), see, *e.g.*, pages 362-363. For several years thereafter, others agreed that, at the time, there were many reasons for one skilled in the art to doubt that Fire's method could or would be effective, as claimed in the '559 patent in a human cell (see, e.g., Paddison *et al., Proc. Nat'l Acad. Sci.* 99(3):1443-1448 (Feb. 5, 2002) (previously cited in recorded arguments).

The previously cited statements are fully supported by the Examiner's newly cited reference, PCT publication WO 00/44895, at page 2, lns 14-25, Kreutzer states that:

> Aus Fire A. et al., NATURE, Vol. 391, pp. 806 ist es bekannt, daß dsRNA, deren einer Strang abschnittsweise complementär zu einem zu hemmenen Gen eines Fadenwurms ist, die Expression dieses Gens mit einer hohen Wirksamkeit hemmt. Es wird die Auffassung vertreten, daß die besondere Wirksamkeit der verwendeten dsRNA in Zelles des Fadenwurms nicht auf dem AntiSinn-Prinzip beruht, sondern möglicherweise auf kataytische Eigenschaften der dsRNA bzw. durch sie induzierte Enzyme zuruckzuführen ist. -- Über die Wirksamkeit spezifischer dsRNA in bezug auf die Hemmung der Genexpression, insbesondere in Säugerzellen und humanen Zellen, ist in diesem Artikel nichts ausgesagt.

In US Publ. Patent Appl. 2005/0100907, published May 12, 2005 Kruetzer states at paragraph 0005 that:

> [0005] It is known from Fire, A. et al., NATURE, Vol. 391, pp. 806 that dsRNA whose one strand is complementary in segments to a nematode gene to be inhibited inhibits the expression of this gene highly efficiently. It is believed that the particular

> activity of the dsRNA used in nematode cells is not due to the antisense principle but possibly on catalytic properties of the dsRNA, or enzymes induced by it. -- Nothing is mentioned in this paper on the activity of specific dsRNA with regard to inhibiting the gene expression, in particular in mammalian and human cells.

This same paragraph is reiterated by Kruetzer in US Publ. Patent Appl. 2004/0102408, published May 27, 2004; in US Publ. Patent Appl. 2004/0072779, published April 15, 2004; and in US Publ. Patent Appl. 2004/0053875, published March 18, 2004.

In US Publ. Patent Appl. 2004/0175703, published September 9, 2004, Kreutzer states at paragraph 0006 that:

> [0006] WO 99/32619 (Fire *et al.*) discloses the use of a dsRNA of at least 25 nucleotides in length to inhibit the expression of a target gene in *C. elegans*. dsRNA has also been shown to degrade target RNA in other organisms, including plants (see, *e.g.*, WO 99/53050, Waterhouse et al.; and WO 99/6163-1, Heifetz *et al.*) and Drosophilia (see, *e.g.*, Yang, D., *et al., Curr. Biol.* (2000) 10:1191-1200). Despite successes in these organisms, until recently the general perception in the art has been that RNAi cannot be made to work in mammals. It was believed that protocols used for invertebrate and plant systems would not be effective in mammals due to the interferon response, which leads to an overall block to translation and the onset of apoptosis (see, *e.g.*, Wianny, F., *et al., Nature Cell Biol.* (2000) 2:70-75; Fire, A., *Trends Genet.* (1999) 15:358-363; and Tuschl, T., *et al., Genes Dev.* (1999) 13(24):3191-97). At least one group of scientists believed that RNAi could only be made to work in mammals if the PKR response could be neutralized or some way avoided, although no suggestions were given as to how this might be achieved (Fire, Trends Genet (1999), *supra*; and Montgomery and Fire, *Trends Genet.* (1998) 14:255-258). However, WO 00/44895 (Limmer) demonstrated for the first time that dsRNA can induce RNAi in mammalian cells, provided that the dsRNA meets certain structural requirements, including a defined length limitation. (emphasis added).

In other words, as Applicant has demonstrated to the Examiner with numerous published articles, one of ordinary skill in the art would not believe that RNAi - in accordance with Fire's teaching in nematodes - would work in mammals. Moreover, they would be led away from practicing the invention taught in the Fire '559 patent in humans. This is not an unsupported conclusion, but rather one the Applicant has proven has been independently made by many who are skilled in the art, now including Kreutzer, in the Examiner's own citation.

Yet the Examiner says that one of ordinary skill at that time of the Fire invention would be taught by the '559 patent how to inhibit expression of a target gene, not only in an invertebrate cell, but in all cells, including human cells. This reads far more into the '559 patent than was actually in the inventor's possession at the time of his invention or that he could have

known or enabled. This is especially true, given Fire's own subsequently published words questioning the effectiveness of his invention in a mammalian cell.

Fire's claim 1 is, in fact, silent on the species of cells for which his method is effective, although dependent claims specify an "animal" - without saying "vertebrate" or "non-vertebrate," and subsequently clarifies that the cells are from a plant, invertebrate and nematode. While the '559 specification states at col. 8, line 16, that "the animal may be a vertebrate or invertebrate" (emphasis added), no other statement teaching or example in the entire application, other than at column 8, addresses any dsRNA response in a cell taken from a "vertebrate," or specifically from a human. Clearly, neither Fire nor his peers believed that the method claimed in the '559 patent would be effective in a mammalian cell without substantial additional experimentation to overcome recognized problems for which the Fire patent remains silent and offers no solution. Thus, the boilerplate lists included in the patent specification were merely wishful-thinking by Fire when the '559 specification was drafted – not a basis for anticipating Applicant's invention under 35 U.S.C. §102(e).

A. Applicant's Response to the Examiner's "Response to Arguments"

In making the Examiner's Response to Arguments regarding this rejection, the Examiner agrees with Applicant that Fire actually teaches a method of using RNAi *only* in invertebrate nematodes, whereas Applicant's invention utilizes RNAi in a target gene in a human cell. Moreover, the Examiner agrees that human cells evoke substantial intracellular defenses to the use of dsRNA, yet such defenses are not found in invertebrate cells. More importantly, neither Fire, nor his peers, offers any discussion or teaching for how to overcome such human cell defenses.

Yet illogically, the Examiner's arguments turn to the enablement of the Fire patent to address whether Applicant's invention is anticipated – this is because he recognizes that the Fire patent *lacks* enablement to support the arguments being made. And that is exactly Applicant's problem in responding to the Examiner – the arguments being made by the Examiner for anticipation of Applicant's invention are not supported by fact in the Fire patent – but only by wishful thinking. Thus, Applicant's Response must necessarily turn to the enablement of the subject matter claimed in the Fire patent, rather than the anticipation rejection itself. This is because the Examiner says that while even a significant amount of experimentation would be required by one skilled in the art equipped with Fire and the state of the art at the time to practice

Applicant's invention – that, in fact, "substantial additional experimentation" is not the same as *undue* experimentation. This is an argument regarding the enablement of the Fire patent – not an argument regarding anticipation.

Anticipation does not involve "undue experimentation." That is an enablement standard, and only an issue when the Examiner tries to construe the claims of the Fire patent to define a broader invention that what is taught and supported in the Fire patent.

However, since the Examiner has elected to discuss what is enabled by the Fire invention, rather than discuss the Fire anticipation of Applicant's invention, Applicant is left with no alternative but to be responsive and discuss enablement of Fire, rather than anticipation under 35 U.S.C. §102(e). Thus, Applicant's Response will focus on the Examiner's errors in making such arguments, and address whether or not Fire enables the use of RNAi in a human cell to disrupt the expression of a mammalian target gene. In arguing that "substantial" experimentation is permitted, Applicant turns to the judicially created patent definitions of "undue" experimentation.

> Factors to be considered in determining whether a disclosure would require undue experimentation have been summarized by the board in *Ex parte Forman*. They include (1) the quantity of experimentation necessary, (2) the amount of direction or guidance presented, (3) the presence or absence of working examples, (4) the nature of the invention, (5) the state of the prior art, (6) the relative skill of those in the art, (7) the predictability or unpredictability of the art, and (8) the breadth of the claims. *In re Wands*, 8 USPQ2d 1400, 1404 (Fed. Cir. 1988)

Addressing the Wands factors in numerical order, the first factor is the amount of experimentation needed. The courts have held that while some experimentation "is not fatal to enablement," so long as "the procedures and cultures are *routine*." This judicial requirement that the permitted experimentation requires the an application of "routine" procedures is a far cry from the Examiner's conclusion that substantial experimentation is permitted before reaching the threshold of reaching impermissible "undue experimentation." Nowhere do the facts suggest that the amount of direction or guidance presented by Fire, which is limited to a biological response of a simple embryonic nematode cell, could or would be translated by one of ordinary skill in the art to sufficient knowledge to practice the invention in a human cell, as is expressly taught by Applicant. Thus, Fire fails to teach a critical element of Applicant's claimed invention.

The systems (invertebrate *C. elegans* nematode vs. mammalian, more specifically human) are radically different, as are the cellular defenses that are evoked by the introduction of

double stranded nucleic acids. In fact, by offering no rebuttal, the Examiner has acquiesced in his Response that "neither Fire nor his peers believed that the [Fire] method would be effective in mammalian cells without substantial experimentation." As a result, not even Fire believed that his invention was enabling for cells - other than the exemplified nematode invertebrate cells. Accordingly, far more than routine procedures would be necessary for one of ordinary skill in the art to adapt the procedures taught by Fire in a lower invertebrate cell - to the operation of effecting RNA inhibition of a target gene in a human cell in the manner taught by Gewirtz.

The presence of working examples in the Fire invention are limited to only cells of *C elegans*, an nematode invertebrate. The prior art at the time of the Fire '599 invention offered no suggestion that nematode results could, or should, be translated into human results. In fact, mammalian data, such as mouse results are often not the same when the results are applied to human cells, see for example, a comparison of the differences between results in mice and in humans by Rangarajan *et al. supra*, emphasizing the unpredictability of this art. Moreover, in this case, as has been previously stated, the prior art cited in the record and acknowledged by the Examiner, recognized the defenses evoked by human cells to the proposed RNAi therapies, and doubted that those defenses could be overcome. The relative skill in the art was and remains high, as previously noted, but even Fire doubted that his own invention could be effective in human cells.

Prior to Applicant's filing date of November 2000, data were never presented by Fire, in the '559 specification or elsewhere, showing the effect of dsRNA on a vertebrate cell. Based upon Fire's subsequent publications, he never tested to ascertain whether dsRNA could inhibit expression in a target gene in a vertebrate cell. While confirmation that an invention would actually function in a mammalian cell may not always be required of an inventor under U.S. Patent Law, some level of proof certainly would have been expected by one of ordinary skill in this art before accepting such a claim as derived from the '559 patent – since it was contrary to the recognized state of the art at the time of the Fire invention in 1998. At that time, and for several years thereafter, the expected response of post-embryonic mammalian cells, was known in the art to be very different from the operation of the '559 invention in an embryonic *C. elegans* cell. Yet the inventor only tested his invention using invertebrate cells. To understand why such behavior is not and should not be permitted, see, *Rasmusson v SmithKline Beecham Corp,* 75 USPQ2d 1297 (Fed. Cir. 2005) ("If mere plausibility were the test for enablement

under section 112, applicants could obtain patent rights to "inventions" consisting of little more than respectable guesses as to the likelihood of their success. When one of the guesses later proved ture, the "inventor" would be rewarded the spoils instead of the party who demonstrated that the method actually worked.")

Fire certainly never made any statement in the '559 specification or later, that would suggest that he believed that his invention would actually work in a vertebrate, mammalian or human cell. Fire or his patent attorney simply included everything possible in his long boilerplate paragraphs – and even then, preceded the list with the indefinite term "may be" because he had no basis for knowing what might work or how to overcome recognized problems in the cells of higher life forms.

Such lists certainly would not have led one of ordinary skill in 1997 or 1998 to attempt to practice the '559 invention in a vertebrate cell, because dsRNA inhibition of a target gene in a vertebrate cell was contrary to the response expected in such cells at the time, particularly when read in the context of published conflicting statements by the inventor himself about the effectiveness of the invention in mammalian cells. In fact, in the '559 specification, Fire failed to even identify the recognized and substantial defense mechanisms that one would have expected in a mammalian cell. Yet these defenses would have made the operation or expected success of dsRNA very different in such a mammalian cell, as compared to the '559 disclosed method of operation of dsRNA in an invertebrate embryonic cell.

The final factor, however, is the breadth of the claims, and therein lies the problem with the Fire reference. Applicant does not question the validity of the Fire '599 patent in the present response, nor has Applicant suggested that the Fire invention as far as the effect in nematodes or invertebrates was not enabled by the *C. elegans* examples. What Applicant questions, however, is the breadth accorded to the Fire claims by the Examiner. Claim 1 of the '599 patent reads:

> 1. A method to inhibit expression of a target gene in a cell *in vitro* comprising introduction of a ribonucleic acid (RNA) into the cell in an amount sufficient to inhibit expression of the target gene, wherein the RNA is a double-stranded molecule with a first strand consisting essentially of a ribonucleotide sequence which corresponds to a nucleotide sequence of the target gene and a second strand consisting essentially of a ribonucleotide sequence which is complementary to the nucleotide sequence of the target gene, wherein the first and the second ribonucleotide strands are separate complementary strands that hybridize to each other to form said double-

stranded molecule, and the double-stranded molecule inhibits expression of the target gene.

In hindsight, perhaps the Fire claim, however, should have been narrowed during prosecution before the PTO to read: "A method to inhibit expression of a target gene in an invertebrate cell *in vitro* . . . ." because the patent fails to teach or suggest anything broader than a method for use in an invertebrate cell, or perhaps more specifically only in a *C. elegans* cell. But that is not Applicant's decision to make, nor is this the forum in which that decision can be made.

What is clear, however, is that *all*, including Fire in his own later publications, agree that the '599 patent certainly never taught or enabled the use of the claimed invention in *all* of the species listed in the boilerplate sections of the patent – and it certainly never enabled use of the invention in a human cell. See, previously discussed citations attached hereto at Tabs 3-7. Therefore, even if the term "invertebrate" is missing from the claims, the Fire patent can reasonably be read *only* to encompass subject matter within what was the subject matter taught or suggested by the '599 invention. Without such a limitation, claims would be indefinite and could never be used to define the boundaries of the invention, thereby permitting others to invent improvements over what has already been patented.

The courts have determined that claim terms will not be given their ordinary meaning when the terms are used in the written description of the patent in a manner that makes it manifestly clear that the ordinary meaning of the claim's terms should be restricted. *See, Teleflex, Inc. v. Ficosa N. Am. Corp.*, 299 F.3d 1313, 1327 (Fed. Cir. 2002) (holding that a claim term may be limited by the specification "via other routes" than the inventor choosing to be his own lexicographer and where claim terms lack sufficient clarity to permit the scope to be ascertained), reh'g and reh'g *en banc* denied 2002 U.S. App. LEXIS 19256 (Fed. Cir. Aug. 30, 2002). *See* also, *SciMed Life Sys., Inc. v. Advanced Cardiovascular Sys., Inc.*, 242 F.3d 1337, 1344 (Fed. Cir. 2001) (finding that the claim must be narrowed because "[t]his is therefore a clear case of disclaimer of subject matter that, absent the disclaimer, could have been considered to fall within the scope of the claim language").

As pointed out by the Examiner in his Response to Arguments – "Applicant's arguments with regards to what Fire understood are not persuasive because there is no way to determine definitively what Fire 'understood' or did not understand" in the '599 patent (emphasis added).

This statement alone shows that the Examiner recognized the indefiniteness of the Fire '599 patent, and demonstrates why Fire cannot be read to encompass more than is enabled by the specification.

B. Applicant's Response to the Examiner's "Response to the Declaration of Dr. Alan Gewirtz

The Examiner has stated that the following of Applicant's additional arguments of record regarding Fire were considered, but were not found to be persuasive:

1) Regarding Applicant's argument that RNAi inhibition of a target gene in a human cell as demonstrated in Applicant's invention was an unexpected result based upon Fire. In response the Examiner states that "an argument of unexpected result is insufficient to overcome a rejection under 35 U.S.C. §102 based upon anticipation." This is an error by the Examiner because a teaching of a method that provides an "unexpected" result is the very definition of a novel invention. If the Examiner agrees that Applicant's method teaches and claims an "unexpected result," then it would follow logically that the Examiner agrees that Applicant's invention is, indeed, novel.

2) Regarding Applicant's argument regarding the validity of the Fire '599 patent. Applicant, however, does not argue the validity of the Fire patent in this forum. Applicant merely points out that the Examiner should not be reading the Fire claims as defining an invention that is broader than that which actually was the subject matter claimed in the patented invention. Nor should the '599 claims be read as encompassing more subject matter than was intended or enabled by the narrow Fire disclosure of RNAi in an invertebrate.

3) Regarding Applicant's argument that Fire failed to teach how to overcome mammalian defenses. In response, the Examiner states that Fire overcame the difficulties by controlling the level of dsRNA, to which Applicant states that the Examiner's conclusion is an error. Nowhere in the '599 patent are the defenses evoked in a human cell discussed or addressed. Fire may use reduced levels of dsRNA, but there is no correlation provided in Fire or in the art at the time of the Fire invention that suggests that reducing the level of dsRNA would enable the claimed invention to operate in a human cell. If such statements are suggested by Fire in the '599 patent, Applicant asks that those lines be pointed out and the clarity of the Fire statements made clear in this regard. The Examiner states that the PKR response in a human cell need not have been understood "in minute detail" by Fire, whereas

in fact, Fire never mentions that he knew of or even considered such a response at all. Where are the Examiner's facts to support what Fire knew, or did not know, when making sweeping statements of this type?

4) Regarding Applicant's argument that Fire's own publications after the patenting of his invention "would actually discourage one of ordinary skill in the art from attempting to practice the '599 invention in vertebrate cells." The Examiner states that Applicant's argument is "countered by the known problem and offering a teaching to overcome this problem, as set forth above." Yet the Examiner is unable to offer any fact supporting such a conclusion, whereas Applicant has provided column and line citations of the published statements to that effect by Fire and by others (see, *e.g.*, Paddison, Svoboda, Wianny, each of which are found unconvincing by the Examiner), and now the addition of a number of patent applications by Kreutzer (Tabs 8-15). Thus, the conclusion must be impermissibly based upon personal and unsupported conclusions, since there is no reason provided by the Examiner to question the published art by recognized authorities in the field following Fire's disclosure of his invention in nematodes.

The Examiner simply says that there was no reason for Fire to state "what was known in the state of the art." While that may be true, it is a red herring when applied to this situation. It is impermissible for the Examiner to read into the '599 patent that the inventor intended to resolve an issue in a mammalian cell when he designed the nematode experiments – while not only failing to discuss the resolution, but also failing to even mention or acknowledge the existence of a major problem in human cells. The reason is much more straight-forward. Fire was not even aware of the problems that would be encountered if dsRNA were used in accordance with his disclosed method to target a gene in a human cell, nor did he know how to overcome it – which is why Fire didn't mention such a problem – let alone discuss it in minute detail. The Examiner's rejection is unsupported and unsubstantiated, and it is unrelated to a summary of "what was known in the art". An inventor must teach how to make and use the invention – yet no evidence supports the conclusion that Fire knew how to use his invention in a human cell – to the contrary he published to others that he questioned it is effective use in such mammalian cells. See, Fire papers at Tabs 3 and 4. Fire taught and enabled only the use of dsRNA in embryonic invertebrate nematode cells.

5) Regarding Applicant's argument that Fire's own publications following the patenting of the Fire invention - raise questions that his invention could not operate in mammalian cells. The Examiner states that Applicant has failed to provide evidence to support this claim. However, to make such a statement means that the Examiner has selectively omitted the lines cited by Applicant in multiple publications by column and line number. See Applicant's record, citing several places where Fire and others have stated that the Fire invention may not function in human cells as described in *C. elegans* invertebrate cells. See attached publications at Tabs 3-15.

In light of the foregoing the Examiner is unable to support a factual basis why the Fire '599 patent or the art surrounding the patenting of the Fire invention should be read to cover the breadth of subject matter suggested by the Examiner. Thus, one of ordinary skill in the art reading the Fire method in an invertebrate cell would not have been led to practice the Fire invention in a human cell, as is expressly taught by Applicant. Thus, Fire fails to teach a critical element of Applicant's claimed invention. In fact, quite to the contrary, a method for disrupting target gene expression at the mRNA level in a human cell, wherein the method comprises initiating RNA interference (RNAi) *in vitro* was neither known, nor suggested, until Applicant's invention in 2000, and thus could not have been anticipated by Fire or any other prior art publication. Accordingly, Applicant respectfully requests that this rejection under 35 U.S.C. §102(e) be reconsidered and reversed..

5. Claim Rejection - 35 U.S.C. §103(a).

The Examiner has for the first time rejected claims 23-27 under 35 U.S.C. §103(a) as being unpatentable over Fire *et al.* ("Fire"), in view of Gewirtz *et al.* (WO 92/19252) ("Gewirtz").

In making this rejection, the Examiner states that it would have been *prima facie* obvious to one of ordinary skill in the art, at the time of Applicant's invention to practice a method of inhibiting the expression of the oncogene cKit *in vitro*, in human leukemia cells (as taught by Gewirtz) using a 25bp double stranded RNA to initiate RNA interference, wherein the dsRNA was comprised in pharmaceutical composition (as taught by Fire) because:

1) according to Gewirtz, "antisense inhibition [by dsDNA] of c-Kit was taught in the prior art as inhibiting the expression of KitR in human leukemia cells;"

2) Fire taught that "dsRNA can be used to initiate RNA interference *in vitro* by targeting oncogenes in human cells, including leukemias;" and

3) Fire taught that "dsRNA used to inhibit gene expression has advantages in the stability of the material to be delivered."

However, in making these statements in the paragraph bridging pages 14 and 15, the Examiner has not only misrepresented what was taught to one of ordinary skill in the art by the cited references, the Examiner has further drawn improper conclusions based upon what the references actually do teach. Moreover, even if combined as suggested, the cited references in combination fail to teach one of ordinary skill in the art how to make and use Applicant's invention as set forth in claims 23-27 with a reasonable expectation of success in light of the knowledge in the art at the time of Applicant's invention.

In order to establish a prima facie case of obviousness three basic criteria must be met. First, there must be some suggestion or motivation either in the references themselves or in the knowledge generally available to one of ordinary skill in the art, to modify the reference or to combine reference teachings. Second, there must be a reasonable expectation of success. Finally, the prior art reference (or references when combined) must teach or suggest all of the claim limitations – in the present situation including the use of the method for disrupting mammalian target gene expression at the mRNA level in a human cell, wherein the method comprises initiating RNA interference (RNAi) *in vitro*. "The teaching or suggestion to make the claimed combination and the reasonable expectation of success must both be found in the prior art, not in the applicant's disclosure." *In re Vaeck*, 947 F.2d 488, 493, 20 USPQ2d 1438, 1442 (Fed. Cir. 1991).

Further, in determining the differences between the prior art and the claims, the question under 35 U.S.C. §103(a) is not whether the differences themselves would have been obvious, but whether the claimed invention as a whole would have been obvious. See, *e.g. In re Hirao*, 535 F.2d 67, 190 USPQ 15 (CCPA 1976).

Applicant respectfully submits that the Examiner has erred in rejecting claims 23-27 under 35 U.S.C. §103(a) as being unpatentable over Fire *et. al.* (AP) ("Fire") as applied in the §102(a) rejection of claims 1, 2, 5, 7-9, 11 and 21-22 above, in view of Gewirtz *et al.* (WO 92/19252) ("Gewirtz"). As a preliminary matter, it is submitted that taken as a whole, the combination of Fire and Gewirtz fails to disclose each and every element of claim 23, or the

claims dependent thereon (claims 24-27). Further, even if the combination of Fire and Gewirtz were to disclose each element of claim 23, it is submitted that the Examiner has not succeeded in demonstrating motivation to modify or combine references as suggested, and also has not provided evidence supporting a reasonable expectation of success in producing, using the referenced invention, a method for disrupting target mammalian gene expression at the mRNA level in a human cell, wherein the method comprises initiating RNA interference (RNAi) *in vitro* as recited in present claim 23.

A. The Obviousness Rejection over Fire.

Applicant respectfully submits that Fire teaches a process fundamentally different from that of present Claim 23 and provides no motivation for modification or combination with the other reference cited by the Examiner. Notably, claim 22 on which claim 23 depends was not cited by the Examiner in the §103(a) rejection. Claim 23 reads:

> The method of claim 22, wherein the method further comprises providing to a population of the human cells; an effective amount of KdsRNA to initiate RNA interference; thereby effecting disruption of target gene expression of KitR at the mRNA level.

However, claim 23 cannot be read in a vacuum as proposed by the Examiner, and as a dependent clam, it also includes all of the limitations of claim 22, which reads:

> 22. A method for disrupting expression of a mammalian target gene *in vitro* at the mRNA level in a human cell, wherein the method comprises providing small interfering RNA guide sequences which are homologous to a portion of the target gene, such that RNAi of the target gene is induced.

Therefore, it is impermissible to ignore the express limitations of claim 22 in claim23 – specifically, that the claimed method provides that "RNAi of the target gene is induced" in a *human* cell. For the foregoing reasons and the reasons of record, the cited Fire patent provides teaching of RNAi only *in vitro* in an embryonic invertebrate cell. Consequently, the Fire '559 patent lacks any direction for one of ordinary skill in the art to practice the claims invention in the cell of any higher life form, and is silent as to how one would overcome the known cellular defenses that would arise in a mammalian or human cell if one were to attempt to practice the '599 patent in a mammalian or human cell. Moreover, later publications by Fire *et al.* and others published their independent beliefs that RNAi would probably not work in a mammalian cell, at least until someone discovered how to overcome the problem of the evoked cellular defenses. See, *e.g.*, the publications referenced at Tabs 3-15.

The problem the Fire failed to address that prevents the use of the '599 invention in human cells is further elucidated by two additional patent applications by Kreutzer *et al.*, US Publ. Patent Appl. 2004/0038921, published February 26, 2004; and US Publ. Patent Appl. 2003/0190654, published October 9, 2003, which in each at paragraph 0004, states:

> [0004] . . . . Unfortunately, the use of long dsRNAs in mammalian cells to elicit RNAi is usually not practical, due to the deleterious effects of the interferon response, as well as the problems associated with the intracellular delivery of large molecules.

As a result, Claim 23 is patentably distinct from the Fire invention for at least the reasons stated above with regard to the rejection under §102(a). Claim 23 recites a process, and must be considered *as a whole*. The process recited in claim 23 is a method taught expressly in a population of *human* cells, which comprises an effective amount of KdsRNA to initiate RNA interference; thereby effecting disruption of target gene expression of KitR at the mRNA level. If Fire is used as the basis for the rejection, then it is missing at least two elements specified in Applicant's invention of claim 23:

1) RNAi of the target gene is induced expressly in a *human* cell; and
2) the population of human cells is provided with an effective amount of KdsRNA to initiate RNA interference; thereby effecting disruption of target gene expression of KitR at the mRNA level.

Despite the Examiner's conclusions, Fire quite simply does not, cannot, and should not be read to encompass the use of RNAi methods in a human cell, as Applicant has previously stated and fully supported. As the evidence has shown, one of ordinary skill in the art would not only not have a reasonable expectation of success if the Fire method were to be attempted in a human cell, such an individual would have been taught not to use such a method by Fire himself and by the many others skilled in the art who, prior to Applicant's invention, stated in a variety of publications that the Fire methods would not work in humans, and further gave reasons why it would not work. Accordingly, Applicant agrees with the Examiner's conclusion, albeit for different reasons, that the Fire '599 patent fails to teach each and every element of Applicant's invention defined in claims 23-27, and that unless those deficiencies are supplemented, there can be no basis for a rejection of Applicant's invention under 35 U.S.C. §103(a).

B. The Obviousness Rejection over Gewirtz.

Applicant agrees with the Examiner's summary on page 14 that in the cited reference Gewirtz (WO 92/19252) teaches "[dsDNA] antisense inhibition of c-Kit proto-oncogene expression in human cells and that c-kit [dsDNA] antisense oligonucleotides are particularly useful against leukemia." Applicant further agrees that the c-kit sequence was known in 1987 by Yarden *et al.* However, none of that information has anything to do with Applicant's presently claimed "method for disrupting expression of a mammalian target gene *in vitro* at the mRNA level in a human cell." If the Examiner's purpose for citing the Gewirtz PCT application was only to establish that the c-kit sequence is well known and a reliable measure of gene expression in a human cell, the Examiner could have simply cited Official Notice. Applicant is not claiming the kit receptor gene, or that use of the c-kit gene is novel; rather it is merely a recognized mammalian gene which reliably demonstrates Applicant's claimed "method for disrupting expression of a mammalian target gene *in vitro* at the mRNA level in a human cell."

The fact that the operation of dsDNA antisense molecules are structurally, chemically, and functionally different from the operation of RNAi is well recognized in the art. Just as there are many ways if treating a heart attack – using either streptokinase or tissue plasminogen activator – the two methods use drugs that are structurally, chemically, and functionally different. Thus, the methods are patentably distinct (US patent numbers will be provided if necessary), even though both are directed toward the same purpose.

DNA and RNA are distinctly different nucleotides, having well known structural differences, susceptibility to degradation, function, sources, etc. No one of ordinary skill in the art of molecular genetics would confuse the two. Gewirtz teaches only the use of dsDNA. Applicant's invention uses only dsRNA. The two cannot be combined in Applicant's invention.

Kreutzer et al., US Publ. Patent Appl. 2004/0038921, published February 26, 2004; and US Publ. Patent Appl. 2003/0190654, published October 9, 2003, at paragraph 0004, both say:

> [0004] . . . unlike [dsDNA] antisense, RNAi degrades target RNA in a non-stoichiometric manner.

The structural requirements for ribozyme and dsDNA antisense activity are different from that which is required for RNAi activity. It is known that ribozymes are long single stranded molecules (always longer than 24 bp) that have several short segments that are complementary to a target. Antisense molecules have at least 4 deoxy bases to stimulate RNAaseH degradation of the DNA/RNA hybrid – but this is not found in dsRNA.

One of ordinary skill in the art would never consider that Applicant's invention that teaches disruption at the mRNA level using RNAi could be practiced by, or combined with, the Gewirtz method of using dsDNA antisense molecules.

C. There is inadequate teaching in Fire to permit use of the method in a human cell and no motivation to combine Fire with the dsDNA antisense method of Gewirtz as suggested by the Examiner.

Contrary to the Examiner's conclusion, while Fire may state that there is a need to improve the art by providing additional methods for disrupting the expression, and while Fire may point to advantages of using dsRNA to provide stability of the material to be delivered, no where does Fire suggest that dsDNA antisense methods can be or should be combined with the use of RNAi. Therefore no motivation is provided in Fire that suggests the use of dsDNA antisense molecules, or combination with any reference that teaches the use of dsDNA antisense.

If, in fact, such a statement exists in Fire, Applicant asks the Examiner to provide page and line number citation. Absent motivation in the reference itself, there is no reason why one of ordinary skill in the art would even consider combining two such completely different methods to have any expectation of success in producing Applicant's claimed invention.

D. The combination of Fire and Gewirtz fails to teach every element of claim 23.

Even if Fire were to disclose each and every element of claim 23 (which it does not), and if Fire were to provide motivation to modify its teachings to include the method in a human cell, it lacks sufficient teaching to do so. Further no combination of prior art with Fire would, at the time of Applicant's invention, provide motivation to modify or combine references to lead to the invention of claim 23, which expressly claims use in a human cell. Claim 23 and all claims depending therefrom recite a "method for disrupting expression of a mammalian target gene *in vitro* at the mRNA level in a human cell" by initiating RNAi, which must be considered *as a whole.*

As stated above, modifying the process of Fire by the addition of a method that teaches the use of dsDNA antisense would completely vitiate the core teachings of either Fire in a nematode or Applicant in a human cell. If the proposed modification or combination of the prior art would change the principle of operation of the prior art invention being modified, then the teachings of the reference are not sufficient to render the claims *prima facie* obvious. *In re Ratti*, 270 F.2d 810, 123 USPQ 349 (CCPA 1959).

Modifying the process of Fire by substituting dsDNA antisense molecules for the dsRNA molecules, or combining the two would, therefore, impermissibly change the principle of operation of the Fire method. Therefore, there can be no motivation to modify Fire as suggested by the Examiner.

E. There is no reasonable expectation of success in modifying Fire by adding the antisense method of Gewirtz.

Applicant respectfully submits that the Examiner has provided no evidence that one of ordinary skill in the art would have had a reasonable expectation of success in modifying or combining the Fire reference by the addition of dsDNA antisense technology. The prior art can be modified or combined to reject claims as *prima facie* obvious as long as there is a reasonable expectation of success. *In re Merck & Co., Inc.,* 800 F.2d 1091, 231 USPQ 375 (Fed. Cir. 1986). See also, *In re Vaeck*, 947 F.2d 488, 493, 20 USPQ2d 1438, 1442 (Fed. Cir. 1991) (The teaching or suggestion to make the claimed combination and the reasonable expectation of success must both be found in the prior art, not in the Applicant's disclosure).

If, on the other hand, one is required to turn to a completely different double stranded process to permit the use of the Fire method in a human cell, it proves that there was no RNAi system available prior to Applicant's invention that would function in a human cell. If such an RNAi system were available, the Examiner would not have had to turn to the unrelated dsDNA antisense art – which is completely different in structure, character, function and process from RNA inhibition. Antisense operates differently to produce a different result, and therefore cannot properly be combined with Fire. Applicant respectfully submits that the combination is proposed by the Examiner because there was no reference available in the RNAi art, and suggests that since the Examiner is in an Art Unit at the US Patent and Trademark Office that also examines antisense inventions, he has turned to that art for additional support to provide a basis for using the Fire RNAi invention in a human cell.

Such support in the DNA antisense art, however, suggests a basic scientific lack of understanding of the operation of dsRNA in targeted RNAi interference. One of ordinary skill in the art would not have had any reasonable expectation of success in attempting to practice the Fire method in cells of life forms higher than an invertebrate, such as a human cell, simply because they were aware of DNA antisense methods that were known to be effective in a human cell. To do so would confuse the proverbial apples and oranges. While both of these references

target genes in a cell, and the DNA antisense method is shown in a human cell, Applicant has on the record distinguished the two processes, and has in fact, previously modified claims 1 and 22 (upon which claim 23 depends) to *expressly avoid* the antisense art as previously noted.

The Examiner has, however, failed to provide any rationale for one of ordinary skill in the art to have had a reasonable expectation of success in combining RNAi methods with DNA antisense methods, let alone for utilizing the Fire RNAi method in a human cell with a reasonable expectation of success. To render a later invention unpatentable for obviousness, the prior art must enable a person of ordinary skill in the field to make and use the later invention. *Beckman Instruments, Inc.*, 892 F.2d at 1551; *Payne,* 606 F.2d at 314 (previously part of citation in Applicant's Response to Office Action dated September 19, 2005). Thus, the relevant inquiry is not whether the Fire '599 patent was invalid for lack of enablement, but whether Fire enabled persons skilled in this art to practice RNAi in a human cell. It did not!

While it is agreed that an enabling prior art reference need not be a blue print for Applicant's invention, in the case of the Fire '559 patent there was a recognized reason why one would not at the time of the invention have expected the Fire patented methods to operate successfully in mammalian cells. Yet Fire failed to identify the known problems, and offered no teaching what-so-ever for how to overcome them to permit the claimed method to be effective for use in mammalian cells. Therefore, although a patent reference is entitled to its broadest reading, it is not permissible to later read non-enabled meanings or operability into such claims that involve life forms to which the patent claims are silent, in a specification that fails to address known, anticipated problems to be overcome before the invention could be used in a mammalian cell. This is particularly true when the inventor himself later states that he does not believe the invention can function as claimed.

Accordingly, Applicant submits that the Examiner's rejection of claims 23-27 under 35 U.S.C. §103(a) is improper, and there is no basis for combining Fire with Gewirtz (WO 92/19252) as suggested. Applicant respectfully requests that, in light of the foregoing arguments, that this rejection be reconsidered and reversed.

6. Formalities – Response to Examiner's Objections.

The Examiner has further cited grounds for objection in the Office Action mailed December 29, 2005, which were not previously addressed. In paragraph 14 of the Office Action on page 16, the Examiner has objected to claims 9, 11, 21, 26, and 27 "under 37 CFR §1.75(c) as

being in improper dependent form for failing to further limit the subject matter of the previous claim" upon which it depends.

Although these claims have been repeatedly examined over several years, it is somewhat surprising that at this late date the Examiner is now raising these formalities. However, although Applicant disagrees that the claims as written are not properly limiting, in an effort to advance the case to allowance, each of claims 9, 11, 21, 26 and 27 have been amended to describe the limitation in terms of an additional step of the method on which they depend. In addition, claims 22-24 have been amended so that the term "cell" is uniformly used, rather than the plural, "cells," which previously appeared in some of the claims.

Accordingly, Applicant submits that the objections to the claims are moot.

7. In Conclusion

In light of the foregoing discussion and logical reasoning, Applicant therefore, respectfully requests that the Examiner's rejections of claims 23-27 under 35 U.S.C. §112, second paragraph; claims 1, 2, 5, 7-9, 11, 21-27 under 35 U.S.C. §112, first paragraph, regarding written description; claims 1, 2, 5, 7-9, 11, 21 and 22 under 35 U.S.C. §§102(a) and 102(e); and claims 23-27 under 35 U.S.C. §103(a), all be reconsidered and reversed, and that this case finally be moved to allowance.

Respectfully submitted,

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OPINION

Comparative biology of mouse versus human cells: modelling human cancer in mice

Annapoorni Rangarajan and Robert A. Weinberg

Laboratory mice have represented a powerful experimental system for understanding the intricacy of human cancer pathogenesis. Indeed, much of our current conceptualization of how tumorigenesis occurs in humans is strongly influenced by mouse models of cancer development. However, an emerging body of evidence indicates that there are fundamental differences in how the process of tumorigenesis occurs in mice and humans. What are these species-specific differences and how do they affect the use of mice as models of human tumour pathogenesis?

Mouse models of human disease pathogenesis have become a central part of many types of biomedical research, including cancer research. This is because the laboratory mouse provides the most experimentally accessible mammalian model — one that shares organ systems, systemic physiology and genes with humans. One of the main attractions of mouse models has been their ease of genetic manipulation. The mouse germline can be modified to express activated oncogenes in specific tissues or to knock out (inactivate) tumour-suppressor genes in others[1–3]. These genetic tools represent an attractive experimental system for examining many features of cancer pathogenesis. But how faithful is the mimicry of the human disease in mice? There are both parallels and discrepancies between mouse and human carcinogenesis. This article focuses mainly on highlighting the differences in tumour pathogenesis at the cellular and molecular levels in the two species and indicates certain limitations that are inherent in the mouse models that are used for elucidating the human disease process.

Mice are not small people

Size, age and cancer. Humans are about 3,000 times larger than mice and are formed from a proportionately larger number of cells. Moreover, humans live, on average, 30–50 times longer than mice. Given the continued, lifelong turnover of cells in the bodies of both mammals, this means that humans undergo about 10^5 more cell divisions in a lifetime; that is, 10^{16} versus 10^{11} mitoses. As the risk of genetic damage, including the creation of mutant alleles that lead to cancer, increases in proportion to the number of cell divisions, this means that humans should experience vastly higher rates of cancer incidence. Yet, epidemiological studies reveal that the lifetime risk of developing cancer is comparable in both species. About 30% of laboratory rodents have cancer by the end of their 2–3 year lifespan and about 30% of people have cancer by the end of their 70–80 year lifespan[4,5] (FIG. 1). Moreover, although the incidence of cancer increases with age in both species, 30% of humans clearly do not have cancer by the age of 3 years (FIG. 1). So, a marked decrease in age-specific cancer rates has accompanied the substantial increase in lifespan that has occurred during the past 80 million years of the mammalian evolution that led, via the primate lineage, to humans. This decrease in cancer susceptibility has been accomplished through the development of several distinct antineoplastic mechanisms, many of which are intrinsic to human cells[6].

Cancer susceptibility. The differences in cancer susceptibility between mice and humans must, ultimately, be understood at the levels of the whole organism, the individual organs and their constituent cells. One factor that is likely to have an important role at the organismal level is the basal metabolic rate, which is about seven times higher in mice than in humans[7]. This could markedly affect the levels of endogenous oxidants and other mutagens that are produced as by-products of normal oxidative metabolism. For example, endogenous oxidants are known to cause DNA damage by oxidizing the bases; and mice excrete 18-fold more breakdown products of DNA that has been damaged by endogenous oxidants per kg of body weight than do humans[7,8]. As these oxidants might be responsible for the bulk of DNA damage and accumulated mutations in mammalian cells, this increased oxidation would seem to indicate a significantly higher rate of cumulative DNA damage per cell per unit of elapsed lifetime.

At the organ level, it is well-known that many carcinogens are activated or neutralized quite differently in mouse and human liver. For example, an inverse correlation has been found between the lifespan of a species and the rate of DMBA (dimethyl benz(*a*)anthracene) carcinogen binding to DNA, reflecting the differential rate of activation of the DMBA pro-carcinogen into its actively mutagenic diol epoxide form. So, cultured rodent fibroblasts bind activated DMBA to their DNA very efficiently, whereas cultured human fibroblasts do so much less efficiently. In one set of experiments, comparable exposures of fibroblasts of various species revealed a 25-fold increased binding of DMBA per mg of cellular DNA by rodent cells compared with their human counterparts[9]. This provides only one example of the fact that the rates of metabolic conversion of pro-carcinogens to carcinogens can be vastly different in the two species. At the same time, the detoxification of many other potential mutagens can also occur with greatly differing kinetics.

Tumour spectrum and karyotype. The spectrum of age-related cancers in the two species is also quite different[10]. Whereas many strains of laboratory mice tend to develop cancer in the cells of mesenchymal tissues — such as lymphomas and sarcomas — most age-related cancers in humans arise in epithelial-cell layers and lead to carcinomas (BOX 1). The mechanistic basis for this species-specific difference in the tissues that are affected by cancer is poorly understood; human diet and tobacco use are clearly contributing factors, but biological differences in the human and mouse tissues are also likely to contribute. One possible determinant of these interspecies differences in tissue susceptibility has been indicated by recent work with telomerase-deficient mice[11]. After passing through five or six organismal generations, cells in these mice show telomere collapse and resulting end-to-end chromosomal fusions[12] (FIG. 2). For unknown reasons, the spectrum of tumours that arise in such mice on a *Trp53*-mutant background resembles the epithelial cancers that are typical in humans, rather than the mesenchymal cancers that are usually observed in mice[13]. Although the mechanism(s) behind this profound shift in mesenchymal versus epithelial target-organ susceptibility remains obscure, it is clear that molecular processes that are intrinsic to normal mouse and human cells dictate profound differences in tissue-specific cancer incidence.

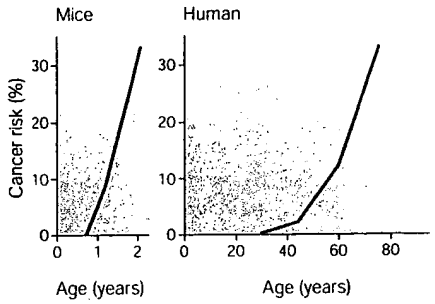

Figure 1 | **Age distribution of cancer in mice and humans.** Mice data are from REF. 61. Human data are from the American Cancer Society, Cancer Facts and Figures 2003 life tables (see online links box).

The cytogenetic profiles of mouse and human tumours present another key difference. Most epithelial carcinomas of humans show highly abnormal karyotypic profiles — characterized by changes in chromosome number and numerous non-reciprocal translocations (NRTs)[14] — whereas these features are uncommon in murine tumours. The mechanistic basis of these differences is also not clearly understood. However, tumours that arise in late-generation telomerase-deficient mice on a *Trp53*-mutant background are characterized by the occurrence of several NRTs[13], indicating that telomere dysfunction might be linked to the generation of NRTs in human cancer cells (FIG. 2).

Despite the overall similarities in organ systems between mice and humans, there are subtle differences with respect to physiology and tissue architecture that can alter the tumour phenotype drastically. For example, humans who inherit one defective allele of the adenomatous polyposis coli (*APC*) tumour-suppressor gene succumb to polyposis of the large intestine at a high frequency because of frequent loss of the surviving wild-type allele in many cells within the colon. The polyps that develop progress to carcinomas and eventually become invasive. However, *Apc*-heterozygous mice tend to develop polyposis of the small intestine and these polyps usually do not progress to yield invasive tumours[15].

Similarly, germline mutations of several human cancer-associated genes that were introduced into the mouse germline yield tumour phenotypes at variance with the human disease[2] (TABLE 1). So, subtle differences in the physiological and biological properties that are intrinsic to the cells of the two species can apparently cause profound changes in their responses to cancer-causing alterations, which in turn can affect neoplastic outcomes.

Intracellular wiring of cells

The process of cellular transformation — by which a normal cell becomes converted into a cancer cell — has been amply studied *in vitro* using cell-culture techniques. Various cultured cells can be subjected to a common set of experimental conditions and the effects of individual genetic alterations or environmental factors can be examined. This potent tool has been instrumental in uncovering important differences between the cellular requirements for transformation of cells from the two species, and we will concentrate on these differences for much of the remainder of this article, because of the implications they hold for the process of tumour progression in human and murine tissues.

Telomeres and telomerase. A principal, long-noted difference between mouse and human cells relates to the observations that murine fibroblasts growing in culture undergo spontaneous immortalization at a high frequency, whereas human fibroblasts fail to do so[16]. Instead of undergoing immortalization, human cells invariably enter into the state of replicative senescence after a defined number of cell divisions *in vitro*[17] (BOX 2). They can remain in this non-proliferating, yet viable, state for extended periods of time. However, in the presence of viral oncogenes, such as the simian virus 40 large T (SV40 LT) oncogene, human cells circumvent senescence, only to succumb later (after 10–20 further population doublings) to a second process termed 'crisis', which is characterized by massive cell death; an immortalized clone might emerge spontaneously and at a low frequency ($\sim 10^{-7}$ per cell division) from a population of cells in crisis[18]. Therefore, replicative senescence and crisis represent two barriers that human cells must overcome on their way to immortalization. As acquisition of immortalized growth seems to be an essential attribute of cancer cells, these differences in immortalization *in vitro* might signal comparable differences that occur during tumorigenesis *in vivo* in the two species.

The relative ease with which cultured murine cells become immortalized in culture can be explained, in part, by differences in the telomere biology of the two species. These structures protect the chromosome ends from degradation, rearrangement and fusion with other chromosomal ends. In cultured human cells, the ends of the chromosomes shorten by 50–100 base pairs with each round of cell division. It has been hypothesized that such telomere erosion in human cells — which leads to a loss of protection of chromosomal DNA ends — triggers cellular senescence and, subsequently, crisis and thereby can limit the replicative potential of human cells[19].

Telomerase — the enzyme that maintains and extends the telomeric ends — is functionally active in most cells of the mouse[20], whereas many adult human somatic cells lack readily detectable levels of this enzyme activity[21]. Furthermore, the telomeres of mice are substantially longer than those of humans (40–60 kilobases in mice versus 10 kilobases in humans), possibly reflective of the continued actions of the telomerase enzyme in post-embryonic murine somatic cells. Consistent with the telomere-erosion hypothesis, restoration of telomerase activity helps to circumvent senescence in some human cell types — such as fibroblasts — and is able to prevent entrance into crisis, thereby promoting immortalization[22]. So, in part because of their normally long telomeres and constitutive expression of telomerase, murine cells readily undergo spontaneous immortalization, whereas human cells must actively acquire a mechanism (usually de-repression of telomerase expression) to prevent telomere shortening and bypass senescence and crisis.

The p53 and RB pathways. Other cellular mechanisms, in addition to telomere biology, are intimately associated with the regulation of senescence. These involve the two dominant cellular tumour-suppressor pathways — p53

Box 1 | **Most common neoplasms**

Humans in the West
- Basal-cell carcinoma of skin
- Breast carcinoma
- Prostate carcinoma
- Squamous-cell carcinoma of skin
- Lung carcinoma
- Colorectal carcinoma
- Urinary bladder carcinoma
- Melanoma
- Uterine carcinoma
- Kidney and renal pelvis carcinoma

Commonly used laboratory mice
- Lymphoma
- Sarcoma
- Leukaemia
- Lung adenoma and carcinoma
- Hepatoma and hepatic carcinoma
- Histiocytic sarcoma
- Fibrosarcoma
- Haemangiosarcoma
- Osteosarcoma
- Leimyosarcoma

and retinoblastoma (RB). These pathways are regulated by, among other proteins, the products of the *CDKN2A* locus, which encodes two structurally distinct proteins — INK4A and ARF — by use of overlapping but distinct reading frames[23]. By inhibiting the cyclin-dependent kinases that are responsible for phosphorylating (and thereby inactivating) RB, INK4A encourages the accumulation of the active, growth-suppressing form of RB, resulting in arrest in the G1 phase of the cell cycle. ARF binds directly to MDM2, resulting in the stabilization of p53, which in turn leads to the upregulation of the CDK inhibitor WAF1 (also known as CIP1 and p21), causing both G1 and G2 arrest. Therefore, a common end point of activating either the p53 or RB pathway is often cell-cycle arrest.

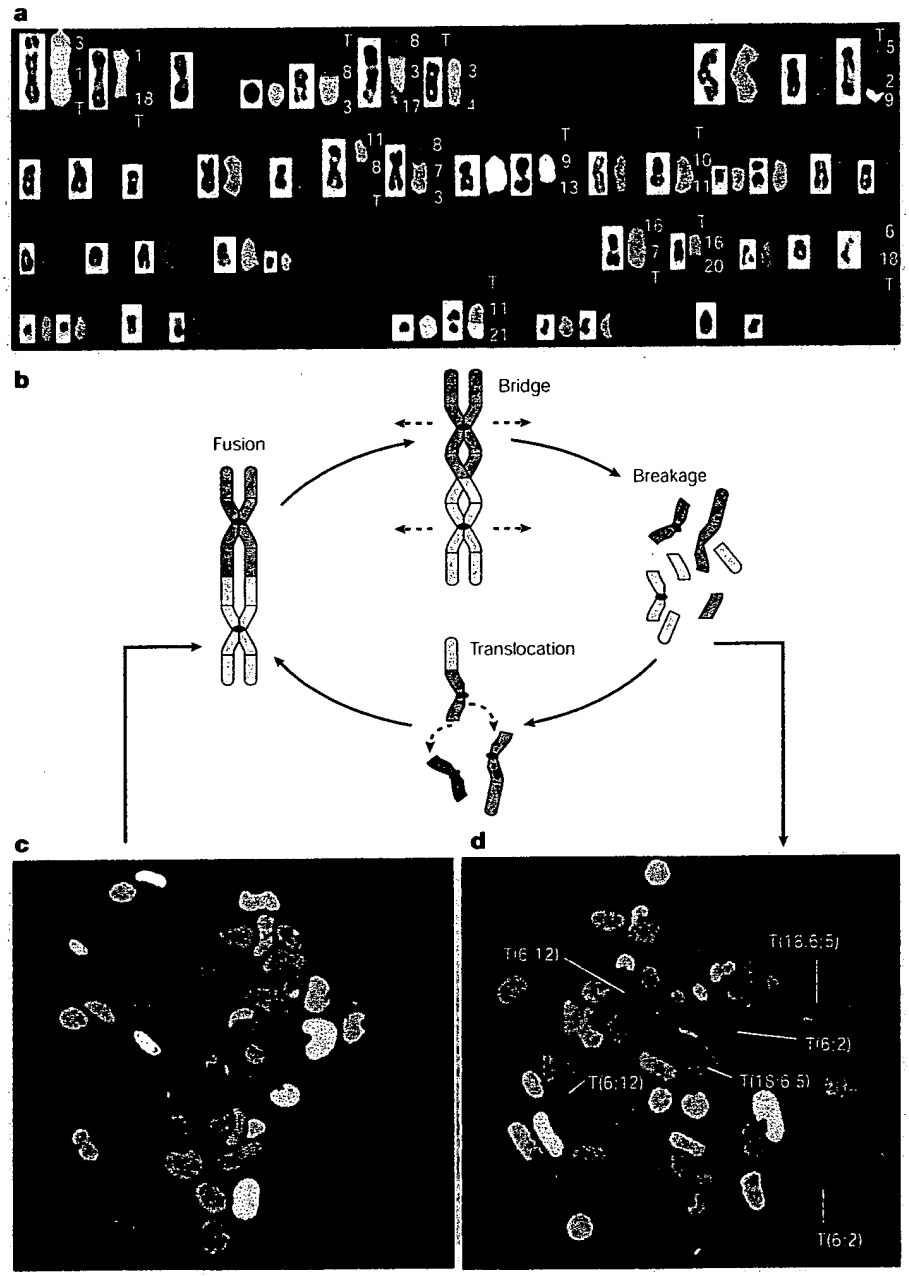

Figure 2 | **Karyotype of mouse and human tumours. a** | Spectral karyotype (SKY) profile of human ovarian epithelial cancer that shows non-reciprocal translocations (denoted T). **b** | The schematic shows telomere shortening leading to breakage–fusion–bridge cycles. **c** | SKY profile of a tumour in early-generation telomerase RNA component (*Terc*)-null mice. **d** | Late-generation *Terc*-null mice have non-reciprocal translocations in tumours. Panel **a** reproduced with permission from REF. 62 © (2002) American Association for Cancer Research. Schematic in **b** adapted and panels **c** and **d** reproduced from REF. 10 © (2000) Nature Publishing Group.

When wild-type mouse embryonic fibroblasts (MEFs) are grown in standard culture conditions, they experience a senescence-like phenotype, apparently triggered by oxidative stress[24]. This stress-induced senescence phenotypically resembles the replicative senescence of cultured human fibroblasts[25]. Introduction of an oncogene such as *RAS* also triggers a senescence-like response in both mouse and human primary cells[26]. Although provoked by distinct physiological signals, these forms of senescence use the same cellular control machinery — involving the p53 and RB pathways — to elicit this response. However, the relative contributions of these pathways to senescence differs in cells of the two species.

The p53 pathway seems to have a predominant role in regulating the senescence of murine fibroblasts, whereas the RB pathway seems to have a principal role in human cells. In response to culture- or *RAS*-induced senescence, wild-type MEFs show an increase in Arf and p53 levels[26,27]. Consistent with this, MEFs from *Arf*-null or *Trp53*-null mice escape senescence[28,29] (FIG. 3). However, *Cdkn1a*-null MEFs fail to escape senescence, indicating that a p53 target other than Waf1 (the product of *Cdkn1a*) is the relevant downstream effector of senescence in murine cells[30]. Senescent MEFs also show an increase in the levels of Ink4a; however, MEFs from *Ink4a*-null or *Rb*-null embryos succumb to both culture- and *RAS*-induced senescence[31–33]. Taken together, these observations indicate that the Arf–p53 pathway is the principal governor of senescence in murine cells. Interestingly, *Rb/p107* double-knockout and *Rb/p107/p130* triple-knockout MEFs have been shown to escape senescence[32,34,35], indicating that the Rb pathway might also contribute to murine cellular senescence, albeit in a more minor way than in human cells (see below).

A contrasting response is seen in human cells. As human fibroblasts approach replicative senescence (or, alternatively, experience an active RAS oncoprotein), the levels of INK4A, but not ARF or p53, increase significantly[36]. Also, cultured human mammary epithelial cells and keratinocytes show upregulation of INK4A and associated growth arrest early on; however, they spontaneously lose INK4A expression, frequently via promoter methylation, and thereby gain a proliferative advantage. Subsequent introduction of telomerase reverse transcriptase (TERT; the catalytic subunit of human telomerase) into these cells enables them to undergo immortalization[37,38]. More recently, it has been shown that dermal fibroblasts that were derived from a human patient with a mutation in both copies of the *CDKN2A*

Table 1 | **Comparison of conventional and modern KO mouse models of human tumour-suppressor genes**

Tumour-suppresor gene	Spectrum of tumours in conventional mouse KO models*	Spectrum of tumours in humans (loss of function)*	Recent genetic modifications	Spectrum of tumours in modified KO models	References
Rb	Brain, pituitary	Retinoblastoma, osteosarcoma	Additional loss of *p107*	Retinoblastoma	53
Trp53	Osteosarcoma, lymphoma, soft-tissue sarcoma	Breast carcinoma, brain, sarcomas, leukaemia	Additional loss of *Terc*	Breast and other carcinomas	13
Apc (*Apc^{MIN}*, *Apc$^{\Delta 716}$*, *Apc$^{\Delta 580}$*)	Multiple polyps in small intestine	Polyps in colon progressing to carcinomas	Conditional colon-specific inactivation	Polyps in colon	63
Ink4a	Fibrosarcoma, lymphoma, squamous-cell carcinoma	Familial melanoma, sporadic pancreatic, brain tumours	Crossed with *Arf$^{+/-}$* mice	Metastatic melanoma, sarcoma, carcinoma, lymphoma	33
Brca1	No tumour susceptibility	Breast, ovary	Conditional mammary-specific inactivation of *Brca1*	Mammary tumours	64
Brca2	No tumour susceptibility	Breast, ovary	Conditional mammary-specific inactivation of *Brca2* and *Trp53*	Mammary tumours	65
Nf1	Pheochromocytoma, myeloid leukaemia	Neural-crest-derived benign neurofibroma and malignant fibrosarcoma	Additional loss of *Trp53*	Neural-crest-derived malignant glioblastoma	66
Nf2	Osteosarcoma, fibrosarcoma, lung adenocarcinoma, hepatocellular carcinoma	Schwannomas, meningiomas, ependymomas, gliomas	Schwann-cell precursor-specific ablation of *Nf2*	Schwannomas	54

*Data from Ref. 2. *Apc*, adenomatosis polyposis coli; *Brca*, breast cancer; KO, knockout; *Nf*, neurofibromatosis; *Rb*, retinoblastoma; *Terc*, telomerase RNA component.

locus (encoding INK4A) — which renders the resulting INK4A protein non-functional — are resistant to oncogenic *RAS*-induced senescence[39]. Observations such as these indicate that INK4A is the key governor of senescence in human cells. However, *TP53* or *CDKN1A*-null human fibroblasts have been shown to escape replicative senescence[40,41]; nevertheless, they succumb to *RAS*-induced senescence, indicating that the contribution of the p53 pathway to human cell senescence is minor compared with its central role in murine cells.

Protein phosphatase 2A. Bypassing senescence represents the breaching of one barrier to experimental transformation of human cells. The ability to maintain telomeres, which can be achieved by ectopically expressed telomerase, represents another. However, the resulting immortalized human cells are still not transformed following the introduction of an oncogene such as *RAS*. Introduction of oncogenic *RAS* into immortalized murine cells, on the other hand, endows them with anchorage-independent proliferation *in vitro* and tumorigenicity *in vivo*[42,43]. This indicates that yet other differences exist between mouse and human cell transformation.

Immortalized human fibroblasts that ectopically express *TERT* together with viral oncoproteins that inactivate p53 and RB (such as human papillomavirus (HPV) E6, HPV E7 or SV40 LT) are still not transformed by oncogenic *RAS*[44,45], but when the entire SV40 early region (instead of a complementary DNA that encodes only the viral large T oncoprotein) was used in transformation experiments, this viral DNA, together with ectopic *TERT* expression, rendered human cells transformable by a subsequently introduced *RAS* oncogene[46]. This indicates that this SV40 genomic construct encodes one or more additional functions that are not specified by the large T cDNA. The additional function is mediated by the viral small T (ST) oncoprotein. In its presence, the already-immortalized human cells can indeed be transformed to a tumorigenic state by a *RAS* oncogene[45,46].

Box 2 | **Senescence *in vitro* and *in vivo***

Leonard Hayflick first reported, in 1961, that lung fibroblasts explanted from a human fetus had a replicative limit of ~50 divisions when passaged serially in culture. Similarly, most mammalian cells have a finite replicative potential *in vitro*, which is referred to variously as the Hayflick limit, replicative senescence or mortality stage 1 (M1). Such cells are irreversibly growth-arrested in the G1 phase of the cell cycle and are no longer responsive to mitogenic stimuli. As these cells are unable to divide, senescence represents a barrier to transformation *in vitro*. Morphologically, senescent cells appear flattened and enlarged, and show senescence-associated acidic β-galactosidase enzyme activity (SA-β-Gal)[55].

Evidence indicates that senescence might well impede tumour formation *in vivo* as well. For example, in skin samples from human donors of different ages, an age-dependent increase in SA-β-Gal activity was observed in dermal fibroblasts and epidermal keratinocytes[55]. Also, cells that are explanted from old donors tend to senesce after fewer population doublings *in vitro* compared with cells from young donors, indicating that senescence is not merely an artefact of *ex vivo* culture but also occurs *in vivo*.

In addition to extensive passaging in culture, several other cell physiological stimuli — such as oxidative stress, DNA damage or activation of an oncogene — can induce a senescence-like growth arrest in cultured mammalian cells. These other forms of senescence, often referred to as stasis, display the same phenotypic characteristics as those of cells undergoing replicative senescence.

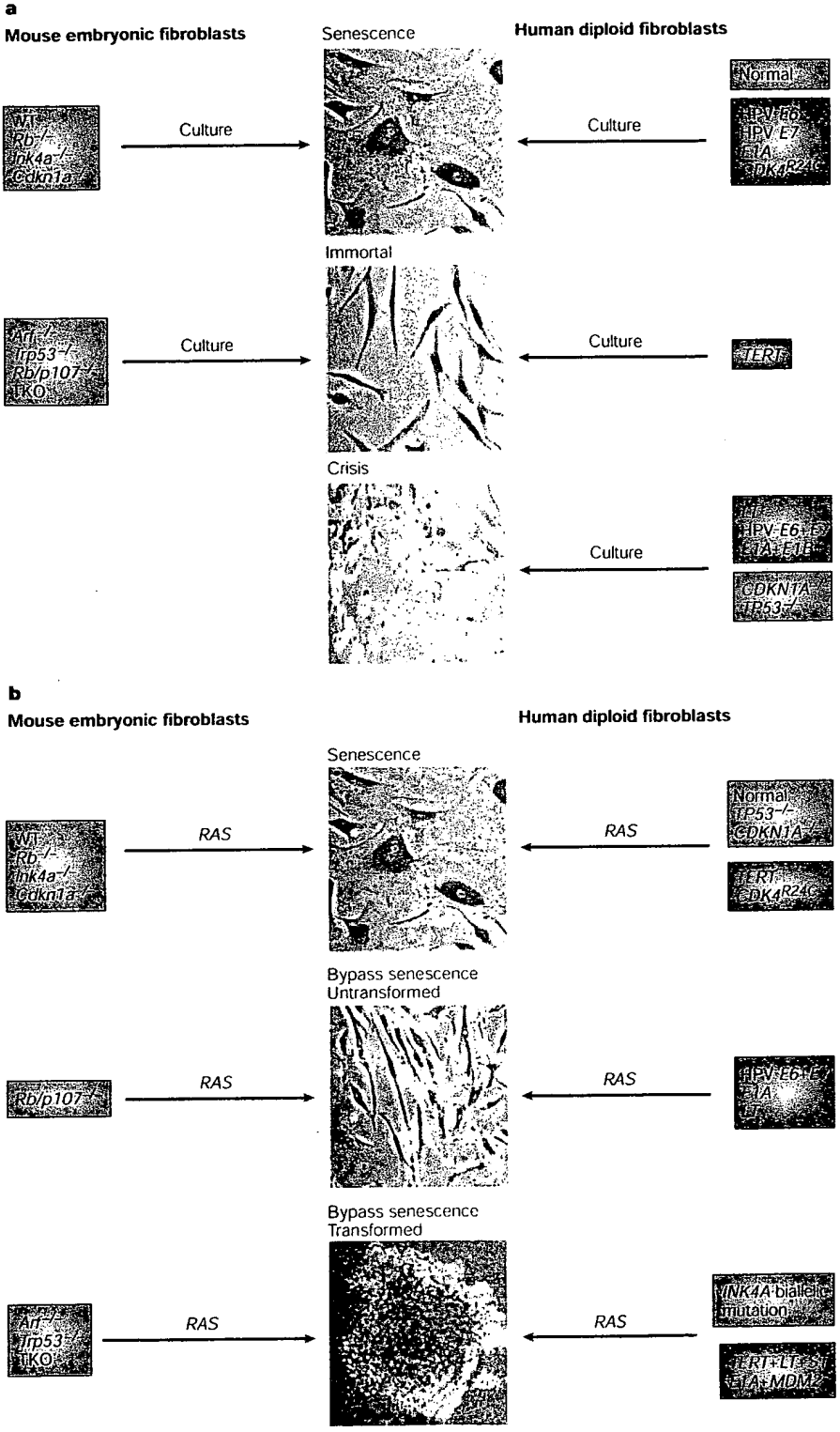

Figure 3 | **Escape from culture- and oncogenic *RAS*-induced senescence.** Effects of various genetic backgrounds (pink boxes) and introduced genes (green boxes) on senescence. **a** | Examines the effects on bypassing culture-stress-induced senescence in mouse embryonic fibroblasts (left) and on bypassing replicative senescence in human diploid fibroblasts (right). **b** | Examines the effects on bypassing Ras-induced senescence in mouse embryonic fibroblasts (left) and human diploid fibroblasts (right) and subsequent transformation. *CDK4*^{R24C}, INK4A-insensitive mutant of cyclin-dependent kinase 4; *CDKN1A*, encodes WAF1 (also known as CIP1 and p21); HPV, human papillomavirus; LT, simian virus 40 large T; *Rb*, retinoblastoma; ST, simian virus 40 small T; TERT, telomerase reverse transcriptase; TKO, triple knockout of Rb, p107 and p130; WT, wild type.

The essential contribution of ST antigen to transformation has been shown to derive from its ability to interact with and inhibit a subset of the many activities of the widely acting cellular enzyme protein phosphatase 2A (PP2A)[45]. PP2A is responsible for the dephosphorylation of many (and possibly a vast throng of) cellular phosphoproteins that are phosphorylated on serine or threonine residues[47]. Recent evidence has shown that constitutive activation of the phosphatidyl inositol-3 kinase (PI3K) pathway and increased expression of c-*MYC* can substitute for ST functions in transforming late-passage human mammary epithelial cells[48], which provides a clue about the specific cellular signalling pathways that are perturbed by ST.

Importantly, the transformation of mouse cells has not been found to be dependent on the inactivation of PP2A or the actions of the small T oncoprotein, indicating yet another distinction in the regulatory circuitries of human versus mouse cells. However, it has more recently been shown that human cells can be transformed in the absence of ST. In one experiment, co-expression of the *CDK4*^{R24C} allele and oncogenic *RAS* in human keratinocytes (with spontaneous acquisition of telomerase activity *in vivo*) was sufficient for generating invasive tumours[49]. In another experiment, co-expression of *E1A*, *MDM2* and oncogenic *RAS* sufficed to transform human fibroblasts[50]. These two experiments, nevertheless, do not rule out the possibility that these cells might have additionally acquired ST-like functions. Alternatively, the requirement of ST might depend on the combination of collaborating oncogenes and tumour-suppressor genes to give rise to a particular tumour type.

The RAS pathway. The RAS proteins are low-molecular-weight G proteins that possess the ability to bind guanine nucleotides. Following activation by upstream receptor tyrosine kinases, RAS proteins exchange their GDP for GTP and become active in signalling. Mutations in *RAS* genes that render their protein products 'locked' in the GTP-bound and constitutively active state have transforming potential[43]. In its active GTP-bound state, RAS binds to and activates several downstream effectors through its effector-binding loop domain (amino acids 32–40; FIG. 4). Three principal RAS effectors that have been shown to have important roles in RAS-mediated transformation are RAF, PI3K and RAL guanine nucleotide exchange factors (RAL-GEFs)[43].

Evidence shows that mouse and human cells differ in their use of Ras-effector pathways for transformation.

Constitutive activation of the Raf–mitogen-activated protein kinase (MAPK) pathway causes tumorigenic transformation of a spontaneously immortalized NIH-3T3 mouse fibroblast cell line, whereas kinase-deficient mutants of *Raf* or its downstream effectors, *Mek* and *Erk*, block Ras-mediated transformation[43]. This has led to the suggestion that the Raf-effector pathway that is downstream of Ras is both necessary and sufficient for tumorigenic transformation of rodent fibroblasts. Consistent with this, expression of effector-loop mutants of oncogenic RAS (FIG. 4) that limit its signalling largely to activating Raf is sufficient for transformation of NIH-3T3 cells[42]. However, activation of the Raf pathway alone is insufficient for the transformation of immortalized rat intestinal epithelial cells[51], indicating that there might be cell-type or species-specific differences in the requirement of Ras signalling pathways.

Similar experiments that were performed in human BJ fibroblasts and human embryonic kidney (HEK) cells immortalized by SV40 early region and human telomerase enzyme revealed striking differences[52]. Activation of RAL-GEF, but not RAF or PI3K, was required for anchorage-independent growth of these human cells, indicating that the signalling requirements downstream of RAS activation might be quite different in mouse and human cells. Unlike oncogenic RAS, however, activation of RAL-GEFs in these human cells failed to initiate tumour formation *in vivo*, indicating that cooperation of other RAS-effector pathways that involve RAF and/or PI3K is required for complete transformation of these human cells.

These various observations show the existence of key differences in the signalling requirements for the transformation of mouse and human cells *in vitro*. In mouse fibroblasts, perturbation of just two signalling pathways — those involving p53 and Raf–MAPK — seems to be sufficient to mediate tumorigenic conversion. In human fibroblasts, perturbation of six or more pathways — those involving p53, RB, telomerase, PP2A, RAL-GEF and one or more additional RAS-effector pathways — seems to be essential for achieving the same outcome. It is worth noting that alterations in the p53, RB, RAS and telomerase pathways are commonly detected in a wide variety of human tumours.

Future directions

The course of tumour progression can be mapped by the nature and number of altered genes that are present within tumour cells. Indeed, many of the gene alterations that are documented to have central roles in multistep tumour progression are known to affect the regulatory pathways enumerated above. Based on the observations that cumulative cancer risk increases by approximately the fourth power of age in both rodents and humans, the average number of steps in carcinogenesis has been indicated to be about the same in these two species[4,5]. However, if transformation of murine cells requires far fewer genetic and/or epigenetic alterations to the cell genome (as described above), then, by necessity, multistep tumour progression occurring in mice must involve far fewer changes and be far simpler than the comparable processes in humans. Stated differently, these discrepancies in the organization of cell-autonomous regulatory pathways must dictate markedly different courses of tumour progression in the two species. Moreover, the substantially greater number of pathways that are required to transform human cells means that most of the cell-autonomous, anticancer protective mechanisms that are present in human cells must have been developed, or at least perfected, during the time since our evolutionary lineage diverged from that of rodents.

Despite these differences, mouse models have proven extraordinarily useful in understanding various aspects of human tumour pathogenesis[1–3] (BOX 3). Furthermore, knowledge of the discrepancies that are associated with specific mouse models of human cancer has been instrumental in improving these models to make them reflect more closely human cancer pathogenesis. For example, the first generation of transgenic mice (in which an oncogenic transgene is widely expressed in all the cells of a murine tissue from early in the animal's life) validated the causal role of specific gene alterations in tumorigenesis, the cooperation of individual genetic alterations and the multistep nature of tumour development. However, these models failed to accurately model the initiation of many sporadic human tumours, which seems to involve the stochastic activation of an oncogene in a single cell that is surrounded by an otherwise normal tissue microenvironment. This has led to the recent development of transgenic mice with regulatable oncogenes, in which transgene expression can be induced in a tissue-specific and time-controlled fashion and can occur stochastically in a small proportion of cells within a target tissue[1,2].

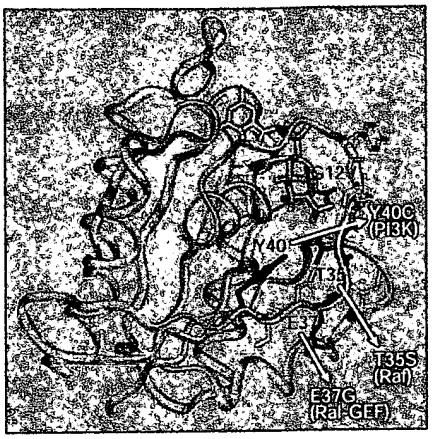

Figure 4 | **Structure of oncogenic HRAS.** The figure shows the ribbon structure of the guanine-nucleotide-binding domain (amino acid 1–166) of oncogenic HRAS G12V bound to a GTP analogue (shown in pink, PDB 521P). The effector loop domain is shown in yellow. Oncogenic HRAS G12V, which additionally harbours the T35S, Y40C or E37G mutation, preferentially binds to and activates RAF, PI3K or RAL-GEFs, respectively.

Conventional mouse knockout models of human tumour-suppressor genes often display a tumour spectrum at variance with the human pathology[2] (TABLE 1). For example, in humans, germline or somatic *RB* gene loss is associated with the development of retinoblastomas and osteosarcomas and, later in life, with small-cell lung carcinomas, whereas mice that have been engineered with *Rb* deletion fail to develop these types of tumours. However, additional deletion of the gene that encodes the Rb-related protein p107 yields retinoblastomas in mice[53]. Similarly, *NF2* mutations in humans lead to schwannomas, whereas conventional *Nf2*-knockout mice develop osteosarcomas. Nonetheless, when a conditional inactivation of both *Nf2* alleles was engineered to occur specifically in the Schwann cells, these mice developed schwannomas[54], indicating that an understanding of the species-specific differences in tumour pathobiology can lead to a better recapitulation of the human disease in mice.

Experiments such as these indicate that it will be desirable in the future to alter the mouse germline by replacing mouse genetic elements with their human homologues, thereby 'humanizing' distinct components of the cellular regulatory circuitry and creating murine cells that increasingly approach the biological behaviour of their human counterparts. Recent technological advances that involve gene-array experiments of human and mouse tumours are likely to

Box 3 | Important lessons learned from mouse models of carcinogenesis

- Cloned candidate human oncogenes that transform cells *in vitro* can trigger cancer *in vivo* in transgenic mice, strongly supporting their role in human tumorigenesis[2,3].
- The deletion of suspected human tumour-suppressor genes from the mouse germline causes tumour susceptibility in these mice, thereby validating these candidate genes as important agents in human carcinogenesis[2].
- Compound mice, which are generated by the interbreeding of mice with specific mutations in oncogenes and tumour-suppressor genes, allow the assessment of how these individual mutations cooperate mechanistically to produce cancer during the course of multistep tumour development[2].
- Ageing telomerase-deficient mice, heterozygous for mutant *Trp53*, show a pronounced shift in their tumour spectra from the mesenchymal cancers that are usually observed in mice, to epithelial cancers with non-reciprocal translocations — features of neoplastic disease in ageing humans. This indicates that differences in telomere length and regulation impacts tumour spectrum and cytogenetics in the two species[13].
- The inactivation of oncogenic transgenes in already-formed murine tumours causes collapse of these tumours, indicating that alterations that are responsible for initiating tumour formation are also essential for tumour maintenance[56].
- Targeted inactivation of the *Nf1* gene in Schwann-cell precursors of mice leads to a neurofibromatosis resembling that seen in humans, which supports the candidacy of these cells as the progenitor cell type in this histologically complex tumor[57].
- Role of stroma in tumour progression: mice that lack mast cells fail to develop certain transgene-induced tumours, indicating that inflammatory cells that are recruited by the tumour contribute in an essential way to tumorigenesis[59]; studies with $Id^{+/-}$;$Id3^{-/-}$ mice show that recruitment of bone-marrow-derived endothelial precursor cells is required for tumour angiogenesis[58,60].

provide additional useful information for the intelligent remodelling of the mouse genome to ensure that its encoded proteins are expressed in ways that more closely resemble the expression patterns of their human orthologues.

Tumour xenograft models that involve the engraftment of human tumours in immunocompromised mice represent yet another model system with which to study certain complex pathobiological processes of human tumorigenesis, such as invasion and metastasis, that are not readily mimicked by the genetically engineered mouse models and that cannot be recapitulated by use of *in vitro* studies. However, interspecies incompatibilities in receptor–ligand interaction between engrafted human tumour cells and the surrounding mouse tissue can contribute to the failure of xenograft establishment and growth. In the case of human carcinoma cells, humanizing the mouse genome with appropriate human stroma-specific genes or humanizing the mouse stroma with human stromal cells before engrafting human cells might substantially improve mouse xenograft models of human cancers.

The various improvements of mouse models of human neoplasias have led to the expectation that mouse models can be exploited not only to understand how human cancers arise, but to generate better anti-tumour therapies. Although we have come a long way since the first mouse models of human cancer were constructed, much needs to be done before it will be possible to reliably predict the behaviour of human tumours from that of their mouse counterparts.

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1. Van Dyke, T. & Jacks, T. Cancer modeling in the modern era: progress and challenges. *Cell* **108**, 135–144 (2002).
2. Herzig, M. & Christofori, G. Recent advances in cancer research: mouse models of tumorigenesis. *Biochim. Biophys. Acta* **1602**, 97–113 (2002).
3. Jonkers, J. & Berns, A. Conditional mouse models of sporadic cancer. *Nature Rev. Cancer* **2**, 251–265 (2002).
4. Ames, B. N., Saul, R. L., Schwiers, E., Adelman, R. & Cathcart, R. in *Molecular Biology of Ageing* (eds Sohal, R. S., Birnbam, L. S. & Cutler, R. G.) 137–144 (Raven Press, New York, 1985).
5. Holliday, R. Neoplastic transformation: the contrasting stability of human and mouse cells. *Cancer Surv.* **28**, 103–115 (1996).
6. Hanahan, D. & Weinberg, R. A. The hallmarks of cancer. *Cell* **100**, 57–70 (2000).
7. Ames, B. N., Shigenaga, M. K. & Hagen, T. M. Oxidants, antioxidants, and the degenerative diseases of aging. *Proc. Natl Acad. Sci. USA* **90**, 7915–7922 (1993).
8. Adelman, R., Saul, R. L. & Ames, B. N. Oxidative damage to DNA: relation to species metabolic rate and life span. *Proc. Natl Acad. Sci. USA* **85**, 2706–2708 (1988).
9. Schwartz, A. G. & Moore, C. J. Inverse correlation between species life span and capacity of cultured fibroblasts to bind 7,12-dimethylbenz(*a*)anthracene to DNA. *Exp. Cell Res.* **109**, 448–450 (1977).
10. DePinho, R. A. The age of cancer. *Nature* **408**, 248–254 (2000).
11. Blasco, M. A. *et al.* Telomere shortening and tumor formation by mouse cells lacking telomerase RNA. *Cell* **91**, 25–34 (1997).
12. Lee, H. W. *et al.* Essential role of mouse telomerase in highly proliferative organs. *Nature* **392**, 569–574 (1998).
13. Artandi, S. E. *et al.* Telomere dysfunction promotes non-reciprocal translocations and epithelial cancers in mice. *Nature* **406**, 641–645 (2000).
14. Atkin, N. B. Lack of reciprocal translocations in carcinomas. *Cancer Genet. Cytogenet.* **21**, 275–278 (1986).
15. Heyer, J., Yang, K., Lipkin, M., Edelmann, W. & Kucherlapati, R. Mouse models for colorectal cancer. *Oncogene* **18**, 5325–5333 (1999).
16. Newbold, R. F., Overell, R. W. & Connell, J. R. Induction of immortality is an early event in malignant transformation of mammalian cells by carcinogens. *Nature* **299**, 633–635 (1982).
17. Hayflick, L. & Moorhead, P. S. The serial cultivation of human diploid cell strains. *Exp. Cell Res.* **25**, 585–621 (1961).
18. Shay, J. W., Wright, W. E. & Werbin, H. Defining the molecular mechanisms of human cell immortalization. *Biochim. Biophys. Acta* **1072**, 1–7 (1991).
19. Harley, C. B. *et al.* Telomerase, cell immortality, and cancer. *Cold Spring Harb. Symp. Quant. Biol.* **59**, 307–315 (1994).
20. Prowse, K. R. & Greider, C. W. Developmental and tissue-specific regulation of mouse telomerase and telomere length. *Proc. Natl Acad. Sci. USA* **92**, 4818–4822 (1995).
21. Kim, N. W. *et al.* Specific association of human telomerase activity with immortal cells and cancer. *Science* **266**, 2011–2015 (1994).
22. Vaziri, H. & Benchimol, S. Reconstitution of telomerase activity in normal human cells leads to elongation of telomeres and extended replicative life span. *Curr. Biol.* **8**, 279–282 (1998).
23. Sherr, C. J. The INK4a/ARF network in tumour suppression. *Nature Rev. Mol. Cell Biol.* **2**, 731–737 (2001).
24. Parrinello, S. *et al.* Oxygen sensitivity severely limits the replicative lifespan of murine fibroblasts. *Nature Cell Biol.* **5**, 741–747 (2003).
25. Sherr, C. J. & DePinho, R. A. Cellular senescence: mitotic clock or culture shock? *Cell* **102**, 407–410 (2000).
26. Serrano, M., Lin, A. W., McCurrach, M. E., Beach, D. & Lowe, S. W. Oncogenic *ras* provokes premature cell senescence associated with accumulation of p53 and $p16^{INK4a}$. *Cell* **88**, 593–602 (1997).
27. Zindy, F. *et al.* Myc signaling via the ARF tumor suppressor regulates p53-dependent apoptosis and immortalization. *Genes Dev.* **12**, 2424–2433 (1998).
28. Kamijo, T. *et al.* Tumor suppression at the mouse *INK4a* locus mediated by the alternative reading frame product $p19^{ARF}$. *Cell* **91**, 649–659 (1997).
29. Harvey, M. *et al.* In vitro growth characteristics of embryo fibroblasts isolated from p53-deficient mice. *Oncogene* **8**, 2457–2467 (1993).
30. Pantoja, C. & Serrano, M. Murine fibroblasts lacking p21 undergo senescence and are resistant to transformation by oncogenic *Ras*. *Oncogene* **18**, 4974–4982 (1999).
31. Sharpless, N. E. *et al.* Loss of $p16^{Ink4a}$ with retention of $p19^{Arf}$ predisposes mice to tumorigenesis. *Nature* **413**, 86–91 (2001).
32. Sage, J. *et al.* Targeted disruption of the three *Rb*-related genes leads to loss of G(1) control and immortalization. *Genes Dev.* **14**, 3037–3050 (2000).
33. Krimpenfort, P., Quon, K. C., Mooi, W. J., Loonstra, A. & Berns, A. Loss of $p16^{Ink4a}$ confers susceptibility to metastatic melanoma in mice. *Nature* **413**, 83–86 (2001).

34. Dannenberg, J. H., van Rossum, A., Schuijff, L. & te Riele, H. Ablation of the retinoblastoma gene family deregulates G(1) control causing immortalization and increased cell turnover under growth-restricting conditions. *Genes Dev.* **14**, 3051–3064 (2000).
35. Peeper, D. S., Dannenberg, J. H., Douma, S., te Riele, H. & Bernards, R. Escape from premature senescence is not sufficient for oncogenic transformation by Ras. *Nature Cell Biol.* **3**, 198–203 (2001).
36. Wei, W., Hemmer, R. M. & Sedivy, J. M. Role of $p14^{ARF}$ in replicative and induced senescence of human fibroblasts. *Mol. Cell Biol.* **21**, 6748–6757 (2001).
37. Dickson, M. A. *et al.* Human keratinocytes that express hTERT and also bypass a $p16^{INK4a}$-enforced mechanism that limits life span become immortal yet retain normal growth and differentiation characteristics. *Mol. Cell Biol.* **20**, 1436–1447 (2000).
38. Stampfer, M. R. *et al.* Expression of the telomerase catalytic subunit, hTERT, induces resistance to transforming growth factor β growth inhibition in $p16^{INK4A}$(-) human mammary epithelial cells. *Proc. Natl Acad. Sci. USA* **98**, 4498–4503 (2001).
39. Huot, T. J. *et al.* Biallelic mutations in $p16^{INK4a}$ confer resistance to Ras- and Ets-induced senescence in human diploid fibroblasts. *Mol Cell Biol* **22**, 8135–43 (2002).
40. Rogan, E. M. *et al.* Alterations in p53 and $p16^{INK4}$ expression and telomere length during spontaneous immortalization of Li-Fraumeni syndrome fibroblasts. *Mol. Cell Biol.* **15**, 4745–4753 (1995).
41. Brown, J. P., Wei, W. & Sedivy, J. M. Bypass of senescence after disruption of *p21CIP1/WAF1* gene in normal diploid human fibroblasts. *Science* **277**, 831–834 (1997).
42. Rodriguez-Viciana, P. *et al.* Role of phosphoinositide 3-OH kinase in cell transformation and control of the actin cytoskeleton by Ras. *Cell* **89**, 457–467 (1997).
43. Campbell, S. L., Khosravi-Far, R., Rossman, K. L., Clark, G. J. & Der, C. J. Increasing complexity of Ras signaling. *Oncogene* **17**, 1395–1413 (1998).
44. Morales, C. P. *et al.* Absence of cancer-associated changes in human fibroblasts immortalized with telomerase. *Nature Genet.* **21**, 115–118 (1999).
45. Hahn, W. C. *et al.* Enumeration of the simian virus 40 early region elements necessary for human cell transformation. *Mol. Cell Biol.* **22**, 2111–2123 (2002).
46. Hahn, W. C. *et al.* Creation of human tumour cells with defined genetic elements. *Nature* **400**, 464–468 (1999).
47. Janssens, V. & Goris, J. Protein phosphatase 2A: a highly regulated family of serine/threonine phosphatases implicated in cell growth and signalling. *Biochem. J.* **353**, 417–439 (2001).
48. Zhao, J. J. *et al.* Human mammary epithelial cell transformation through the activation of phosphatidylinositol 3-kinase. *Cancer Cell* **3**, 483–495 (2003).
49. Lazarov, M. *et al.* CDK4 coexpression with Ras generates malignant human epidermal tumorigenesis. *Nature Med.* **8**, 1105–1114 (2002).
50. Seger, Y. R. *et al.* Transformation of normal human cells in the absence of telomerase activation. *Cancer Cell* **2**, 401–413 (2002).
51. Oldham, S. M., Clark, G. J., Gangarosa, L. M., Coffey, R. J. Jr. & Der, C. J. Activation of the Raf-1/MAP kinase cascade is not sufficient for Ras transformation of RIE-1 epithelial cells. *Proc. Natl Acad. Sci. USA* **93**, 6924–6928 (1996).
52. Hamad, N. M. *et al.* Distinct requirements for Ras oncogenesis in human versus mouse cells. *Genes Dev.* **16**, 2045–2057 (2002).
53. Robanus-Maandag, E. *et al.* p107 is a suppressor of retinoblastoma development in pRb-deficient mice. *Genes Dev.* **12**, 1599–1609 (1998).
54. Giovannini, M. *et al.* Conditional biallelic Nf2 mutation in the mouse promotes manifestations of human neurofibromatosis type 2. *Genes Dev.* **14**, 1617–1630 (2000).
55. Dimri, G. P. *et al.* A biomarker that identifies senescent human cells in culture and in aging skin *in vivo*. *Proc. Natl Acad. Sci. USA* **92**, 9363–9367 (1995).
56. Chin, L. *et al.* Essential role for oncogenic Ras in tumour maintenance. *Nature* **400**, 468–472 (1999).
57. Zhu, Y., Ghosh, P., Charnay, P., Burns, D. K. & Parada, L. F. Neurofibromas in NF1: Schwann cell origin and role of tumor environment. *Science* **296**, 920–922 (2002).
58. Lyden, D. *et al.* Impaired recruitment of bone-marrow-derived endothelial and hematopoietic precursor cells blocks tumor angiogenesis and growth. *Nature Med.* **7**, 1194–1201 (2001).
59. Coussens, L. M. *et al.* Inflammatory mast cells up-regulate angiogenesis during squamous epithelial carcinogenesis. *Genes Dev.* **13**, 1382–1397 (1999).
60. Rabbany, S. Y., Heissig, B., Hattori, K. & Rafii, S. Molecular pathways regulating mobilization of marrow-derived stem cells for tissue revascularization. *Trends Mol. Med.* **9**, 109–117 (2003).
61. Pompei, F., Polkanov, M. & Wilson, R. Age distribution of cancer in mice: the incidence turnover at old age. *Toxicol. Ind. Health* **17**, 7–16 (2001).
62. Bayani, J. *et al.* Parallel analysis of sporadic primary ovarian carcinomas by spectral karyotyping, comparative genomic hybridization, and expression microarrays. *Cancer Res.* **62**, 3466–3476 (2002).
63. Shibata, H. *et al.* Rapid colorectal adenoma formation initiated by conditional targeting of the *Apc* gene. *Science* **278**, 120–123 (1997).
64. Xu, X. *et al.* Conditional mutation of *Brca1* in mammary epithelial cells results in blunted ductal morphogenesis and tumour formation. *Nature Genet.* **22**, 37–43 (1999).
65. Jonkers, J. *et al.* Synergistic tumor suppressor activity of BRCA2 and p53 in a conditional mouse model for breast cancer. *Nature Genet.* **29**, 418–425 (2001).
66. Reilly, K. M., Loisel, D. A., Bronson, R. T., McLaughlin, M. E. & Jacks, T. *Nf1;Trp53* mutant mice develop glioblastoma with evidence of strain-specific effects. *Nature Genet.* **26**, 109–113 (2000).

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Competing interests statement

The authors declare that they have no competing financial interests.

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